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GAMETOPHYTE DEVELOPMENT IN *CHEILANTHES VIRIDIS* VAR. *GLAUCA* (ADIANTACEAE) WITH SPECIAL REFERENCE TO APOGAMY.

Cindy Louise Anderson

A Dissertation Submitted to the Faculty of Science

University of the Witwatersrand, Johannesburg

for the Degree of Master of Science.

Johannesburg 1992.

ABSTRACT

The gametophyte generation of the fern life cycle is initiated with the formation of spores. The spores of *C. viridis* (Forsk.) Swartz var. *glauca* (Sim) Schelpe Anthony are trilete and have a cristate spore wall ornamentation. Under favourable conditions the spores of *C. viridis* var. *glauca* show polar germination.

The prothallial initial gives rise to a uniseriate, elongated germ filament. All the cell divisions are by cross walls perpendicular to the long axis of the filament. Once the germ filament is two to ten cells long, longitudinal cell divisions are initiated. *C. viridis* var. *glauca* follows the *Adiantum* type of prothallial development.

Under the culture conditions used, it was concluded that *C. viridis* var. *glauca* is an obligate apogamous fern. The apogamous bud develops from two to three meristematic cells which occur at the base of the apical notch. Continued development in this area gives rise to the sporophyte. Vascular tissue, a characteristically sporophytic feature, is evident in the primary leaf at an early stage of development. Unlike the sporophytes produced from sexual embryos, no foot region is ever discernible and the root of the sporophyte arises only after a few leaves are well developed.

A darkly staining tannin layer occurs at the level of the tonoplast in the cells at the interface of the gametophte and sporophyte generation. This layer may serve to protect the developing sporophyte from herbivory.

C. viridis var. *glauca* is an obligate apogamous fern therefore both the sporophyte and gametophyte have the diploid chromosome number, in this case, sixty.

This dissertation describes in detail the development of the gametophyte of *C. viridis* var. *glauca* from the time of spore germination to the development of a sporophyte which is able to live independently of the gametophyte.

DECLARATION

I declare that this dissertation is my own unaided work, submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

CINDY LOUISE ANDERSON

TENTH day of AUGUST, 1992.

ACKNOWLEDGEMENTS

I wish to offer my sincere thanks to the following people, without whom this dissertation would not have been possible:-

Professor R.N. Pienaar, Head of the Department of Botany, University of the Witwatersrand, joint supervisor of this study, for his invaluable advice, expertise and encouragement.

Dr M.L. Frean of the Department of Botany, University of the Witwatersrand, joint supervisor of this study, for the valuable discussions on all aspects of this dissertation especially regarding the immunofluorescent study of the microtubules, her patience, encouragement and enthusiasm.

Dr B.M. Parkinson of the Department of Botany, University of the Witwatersrand for sharing her vast knowledge concerning the preparation of fern material for light and electron microscopy and for access to her reference books and reprints.

Dr C. Straker and Dr J. Fletcher both of the Department of Botany, University of the Witwatersrand for the advice and comments they offered during the research commitee meetings.

A final word of thanks to my family and friends for all their encouragement and help throughout the course of this study.

The University of the Witwatersrand and the Foundation for Research and Development are thanked for the bursaries which enabled me to do my MSc.

LIST OF ABBREVIATIONS

aq = aqueous

BSA = bovine serum albumin

C = centigrade

$CaCl_2$ = calcium chloride

$Ca(NO_3)_2.4H_2O$ = calcium nitrate

$Co(NO_3)_2.6H_2O$ = cobalt nitrate

$CoSO_4.5H_2O$ = cobalt sulphate

$CuSO_4.5H_2O$ = copper sulphate

DMSO = dimethyl sulphoxide

DNA = deoxyribonucleic acid

EDTA = ethylenedinitrilotetra acetic acid

EGTA = ethylenebis(oxyethylenenitrilo)tetra acetic acid

ER = endoplasmic reticulum

ETOH = ethyl alcohol

$FeCl_3.6H_2O$ = iron chloride

$Fe(NH_4)_2SO_4.6H_2O$ = iron ammonium sulphate

$FeSO_4.7H_2O$ = iron sulphate

Fig/s = figure/s

FITC = fluoroscein isothiocyanate

g = gram

GA = gibberrelic acid

GTA = glutaraldehyde

H_3BO_3 = boric acid

HCl = hydrochloric acid

HIO_4 = periodic acid

H_2SO_4 = sulphuric acid

IAA = indolacetic acid

K_2HPO_4 = dipotassium hydrogen orthophosphate

KH_2PO_4 = potassium dihydrogen orthophosphate

KNO_3 = potassium nitrate

KOH = potassium hydroxide

kV = kilovolt

M = molar

m = metre

mg = milligram

$MgCl_2$ = magnesium chloride

$MgSO_4.7H_2O$ = magnessium sulphate

ml = millilitre

MLS = multilayered structure

mM = millimolar

μm = micrometre

μmol = micromole

$MnCl_2.4H_2O$ = manganese chloride

$MnSO_4.4H_2O$ = manganese sulphate

MoO_3 = molybdenum trioxide

mRNA = messenger ribonucleic acid

MTSB = microtubule stabilising buffer

N = normal

NAA = naphthylacetic acid

$NaBH_4$ = sodium borate

NaCl = sodium chloride

Na_2EDTA = sodium ethylenedinitrilotetraacetic acid

Na_2glycerophosphate = sodium glycerophosphate

Na_2HPO_4 = sodium phosphate

$Na_2MoO_4.2H_2O$ = sodium molybdate

$NaNO_3$ = ammonium nitrate

$(NH_4)_2SO_4$ = ammonium sulphate

nm = nanometre

OsO_4 = osmium tetroxide

p. = page

PAS = periodic acid - schiff

PIPES = 1,4 - piperazipdia thansulfonsa ure

NNA = ribonucleic acid

rpm = revolutions per minute

s = second

TEM = transmission electron microscope

v = volume

var. = variety

w = weight

$ZnSO_4.7H_2O$ = zinc sulphate

° = degree

% = percentage

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CHAPTER 1

INTRODUCTION

The fern gametophyte is an autotrophic, free-living entity with a definite form, pattern of growth, structure and mode of reproduction (Klekowski and Lloyd, 1968). Compared to the sporophytic generation the prothallus or fern gametophyte is a relatively simple entity, still more or less adapted to aquatic life or to life in a moist environment, lacking vascular tissue and other specialized organs and sometimes surviving for no more than a few weeks (Nayar and Kaur, 1971). These factors have generally made the more complex, highly organized terrestrial sporophyte with vascular tissues, cuticle and stomata, the main choice of study, especially in more recent years (Verdoorn, 1938). The result of this is that the literature present on the gametophyte is rather scattered. There are still large gaps in our knowledge of the development of the gametophyte, especially in the study of the alternative forms of reproduction which a large number of ferns exhibit i.e. apogamy and apospory.

The rationale behind this study was to gain a comprehensive developmental sequence of the fern gametophyte, from the time of spore germination to the formation of the sporophyte. A fern which was known to be apogamous, *Cheilanthes viridis* (Forsk.) Swartz var. *glauca* (Sim) Scheipe and Anthony, was used in this study in an attempt to answer some questions surrounding the formation and development of the apogamous bud and sporophyte. Areas which were studied included:

1) An investigation to establish from which cell or group of cells of the gametophyte the apogamous bud originates,
2) To determine if there is a layer equivalent to the lipophilic layer which delineates the embryo from the rest of the gametophyte in sexually reproducing ferns in *C. viridis* var. *glauca*. Should such a layer exist, then to determine its chemical composition,

3) To determine the order of development of organs from the apogamous bud and to compare these results to ferns that reproduce sexually.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION.

The Pteridophyta are non-flowering spore-producing plants which show an alternation of generations. The ferns exhibit great variety in form and diversity. Most ferns are considered to be shade-adapted species due to their natural habitats, which are generally restricted to shaded forest floors or to other protected micro-environments (Hariri and Brangeon, 1977). The fern used in this study, *Cheilanthes viridis* (Fonsk.) Swartz var. *glauca* (Sim) Scheipe and Anthony, like many of the species of cheilanthoid ferns, grows in rocky places - on ledges and in crevices of cliffs, on talus slopes, at the base of large rocks or in rocky ground. Cheilanthoid ferns are especially numerous in regions where such habitats are common and there is also a dry period during part of the year. They possess a number of morphological characteristics which enable them to withstand these harsh, relatively xeric conditions e.g. complex leaves with many small ultimate segments, or a complex lamina architecture, a densely scaly, pubescent or ceraceous lamina indument, very coriaceous leaf-tissue and abscission zones in the petiole (Hancock and Lucas, 1973; Tryon and Tryon, 1973).

The distinctive characteristics of *C. viridis* var. *glauca* are as follows:-

The varietal epithet *glauca* means "blue-green" and refers to the colour of the fronds. The fronds reach up to thirty three centimetres in length and are held rigidly erect on long, dark stipes which sometimes possess a few brown, hair-like scales but which are more typically smooth. The lamina are lanceolate to triangular in outline and about twice as long as broad. The lamina is bi- or tripinnatifid to tripinnate, at least in the basiscopically developed lowest pinnae. *C. viridis* var. *glauca* has a dark brown, glabrous, grooved rachis. The pinnae are up to eight centimetres long, oblong to lanceolate and obtuse. The ultimate segments are oblong,

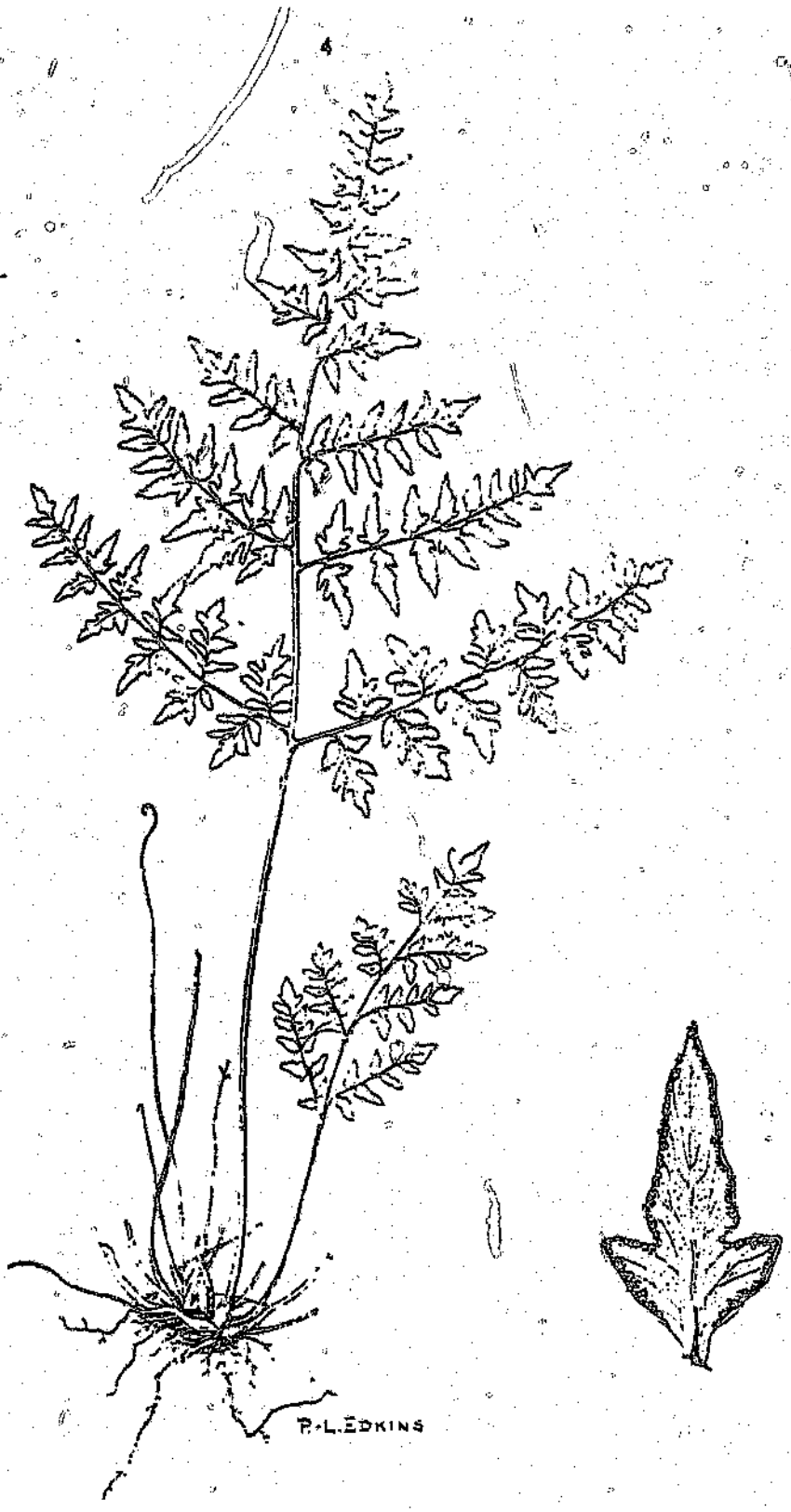


Figure 1:- Diagrammatic representation of *Cheilanthes viridis* var. *glauca* (adapted from Hancock and Lucas, 1973).

obtuse, glabrous with the margins being entire or slightly undulating in sterile members. Venation is distinct and forking. The sori are linear and occur submarginally on the under surface of the segments being borne on the tips of side veins. The sori are confluent when mature, missing the sinuses of the pinnae, and are covered by membranous undulating margins. The spores are trilete. The rhizome is short, creeping and scaly (Jacobsen, 1983; Hancock and Lucas, 1973) (*Fig. 1*).

This fern was formerly known as *Pellaea viridis* (Forsk.) Prantl var. *glauca* Sim and in a recent taxonomic study was transferred to the genus *Cheilanthes* Sw. (Anthony, 1983).

THE LIFE CYCLE OF FERNS.

The life cycle of plants inevitably involves nuclear fusion and, at some subsequent stage, reduction. In ferns this nuclear cycle is often associated with an alternation of two dissimilar generations, one of which, the gametophyte, contains the haploid state of the nucleus, and the other, the sporophyte, the diploid (Bell, 1970).

The Sporophyte Generation.

The conspicuous part of all Pteridophyta, the sporophyte, is a multicellular structure. It has a vascular system for support and for upward and downward conduction of both water and nutrients (Jacobsen, 1983). The fronds are either simple or compound and are attached to the rhizome via the stipe. When mature some of the fronds produce sporangia containing spores. The sporangia usually develop on the abaxial surface of the frond and often form small clusters called sori which may have a protective covering, or indusium, when young (Mickel, 1979; Hancock and Lucas, 1973). An incomplete ring of hardened cells round the edge of the sporangium forms the annulus, between the ends of which are some thin-walled cells termed the stomium. At the time of ripening the annulus absorbs water and straightens out. This action

ruptures the stomium and tears the whole sporangium open with the resultant release of the mature spores (Bastin, 1955).

The Gametophyte Generation.

This generation formed the basis of the study.

The study of gametophyte morphology of ferns has a long history. As far back as 1561, Cordus (quoted in Nayar and Kaur, 1971) noted that all ferns reproduce by means of "pulviscules" borne on the lower surface of their fronds. Subsequent to the development of the microscope in the second half of the seventeenth century, observations on the spores of ferns began to be recorded. The credit for germinating the first fern spore goes to Morison (1699) (quoted in Nayar and Kaur, 1971). The production of ferns by germinating the spores was achieved nearly a century later by Lindsay (1794) (quoted in Nayar and Kaur, 1971). Lindsay was also the first to give a detailed account of spore germination in a fern. The first detailed description of prothallial development in a fern was given by Kaulfuss (1825) (quoted in Nayar and Kaur, 1971). Sachs (1868) (quoted in Nayar and Kaur, 1971) was the first to classify the pteridophytes according to the type of spores they possess (into homosporous and heterosporous) - nearly all classifications to follow used this spore character. By the latter half of the eighteenth century the basic development of the fern gametophyte was well understood (Nayar and Kaur, 1971).

The gametophytic generation starts with the formation of spores in the fern sporangium; the spores germinate to form prothalli, which usually produce sex organs in which, by union of gametes, the second generation of sporophyte is initiated (Towill, 1985; Nayar and Kaur, 1971).

Ultrastructural Details of Fern Spores and the Initiation of Germination.

In dormant spores the protoplasmic contents are surrounded by a thin wall layer. A thick impervious outer layer, the exospore, forms a spore coat. At the proximal pole of the spore the

spore coat has a characteristic trilete or monolete aperture - this is the region where there is a weakness in the spore coat (Nayar and Kaur, 1971).

In the large majority of homosporous ferns, germination is preceded by swelling of the contents of the spores by absorption of water (Nayar and Kaur, 1971; Smith and Robinson, 1969; Pal and Pal, 1963). The place of emergence of the young gametophyte from the spore is largely governed by the organization of the spore wall; the wall is split at the apex of the triradiate ridges in trilete spores and along the ridge representing the line of contact of the four spores in the wedgeshaped (monolete) spores (Verdoorn, 1938).

An attempt to classify the various known patterns of spore germination in homosporous ferns was made by Momose (1942) (quoted in Nayar and Kaur, 1971). He described three types of spore germination:

1) Centrifugal (thallus growing in a direction opposite to that of the primary rhizoid);
2) Centripetal (thallus growing perpendicular to the rhizoid, and rhizoid seated on basal cell of the thallus);
3) Tangential (as in Centripetal, but rhizoid seated at the region of the cross wall between the basal cell and the cell next to it).

Nayar and Kaur (1968) (quoted in Nayar and Kaur, 1971) pointed out that these interpretations of spore germination are incorrect in as much as they ignore the polarity of the germinating spore. Nayar and Kaur (1971) gave a detailed account of the patterns of spore germination, classifying them on the basis of the planes of cell division (in relation to the polarity of the spore) and directions of the primary rhizoid and prothallus. They recognized three categories amongst the homosporous ferns (*Fig. 2*):

1) Polar germination:- The first cell division in the germinating spore is by a wall formed

parallel to the equatorial plane of the spore; elongation of the primary rhizoid and young thallus is parallel to the polar axis of the spore.

2) Equatorial germination:- The first cell division is by a wall formed parallel to the polar axis of the spore, and elongation of the thallus is in a plane parallel to the equatorial plane of the spore.

3) Amorphous germination:- Occurs rarely and exhibits no polarity either with regard to cell divisions or direction of growth resulting in a mass or plate of cells in which a meristematic cell is differentiated at a later stage in one of the peripheral marginal cells.

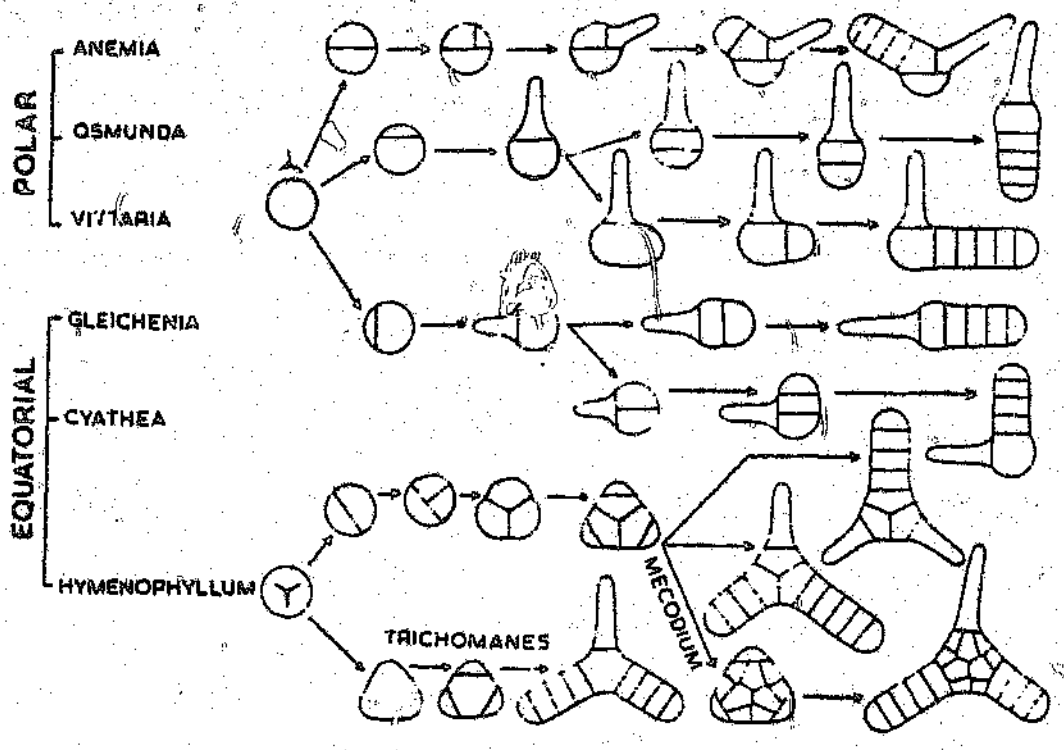


Figure 2:- Schematic representation of types of spore germination in homosporous ferns (adapted from Nayar and Kaur, 1971).

For the fern spore to germinate there should be an adequate supply of moisture, a suitable temperature (20°-28°C) and pH range (pH 4-8) and availability of light of a suitable intensity and quality (Dyer, 1979; Nayar and Kaur, 1971).

The fern spore has a solitary, usually centrally placed nucleus (Miller, 1985) with a single nucleolus surrounded by vacuolate cytoplasm in which are suspended proplastids and food material (Gantt and Arnott, 1965). Food stores are essential since germination and early development of the gametophyte depend upon the utilisation of endogenous food reserves to supply energy and carbon skeletons needed for growth (Raghavan, 1985; Towill, 1985). Fern spores probably contain a variety of reserve materials and a complex of regulatory mechanisms that are involved in their breakdown and utilization since no single reserve material is capable of providing the spore with materials and energy necessary for germination and differentiation of the gametophyte. Metabolic processes in the fern spore are tightly controlled and the sequential hydrolysis of storage reserves provides necessities for particular developmental and physiological activities (DeMaggio and Stetler, 1985).

In *Onoclea* Sw. and *Matteuccia* Sw. lipid constituted twenty seven percent of the spore weight, while in *Dryopteris* Adans. approximately forty percent of the spore was lipid. During imbibition these lipids were hydrolysed and at germination the content had decreased by twelve and a half percent in *Onoclea* and *Matteuccia* and almost fifty percent in *Dryopteris*. It was generally concluded that breakdown of lipids could provide the spore with energy and materials needed for early cell development (DeMaggio and Stetler, 1985; Cran, 1979; Nayar and Kaur, 1971).

In the dormant spore protein granules are abundant but during imbibition and germination the protein content decreases significantly, attaining a steady state that coincides with the emergence of the rhizoid and prothallial cell. Hydrolysis of storage proteins to peptides and constituent amino acids provides the spore with the building blocks to synthesize new proteins for the biogenesis of membranes and organelles and for the production of differentiated cells of the gametophyte (DeMaggio and Stetler, 1985; Gantt and Arnott, 1965).

The proplastids found in the dormant spores are simple structures consisting of a circular profile approximately one micrometre in diameter with a few invaginations of the inner membrane of the boundary envelope or with a few internal tubules. At germination the proplastids have disappeared and fully developed chloroplasts are present in the spores (Cran, 1979). After exposure to light for a short period (i.e. germination has been initiated) starch synthesis in the spore increases considerably - this increase is believed to be associated with the initiation of photosynthesis (Cran, 1979).

As germination progresses a distinct cytoplasmic polarity is imposed upon the cell with the large lipid droplets remaining in the posterior part while the anterior region is occupied by small vacuoles and other cytoplasmic components (Cran, 1979).

The nucleus of the dry, nonchlorophyllous fern spore is rather poorly defined and generally only consists of a mass of chromatin that does not show granularity or internal structural details. Only after hydrolysis of storage reserves has commenced does the nucleus become granular and come to resemble a normal nucleus in its structure (Raghavan, 1985). During the early stages of germination the nucleus of the spore moves from a central location to the anterior end where it divides (Miller, 1985; Gantt and Arnott, 1965).

Nuclear division is followed by an unequal division of the protoplast resulting in the formation of a large prothallial cell and a small rhizoidal cell. Asymmetrical cell division is a critical event in the initiation of cellular differentiation. Bünning (1958) (quoted in Miller, 1985) made the generalisation that without some type of asymmetry, no cellular differentiation could occur. The reasoning behind this view starts with the knowledge that daughter nuclei from a mitosis are genetically identical. The cytoplasmic environment of the nucleus determines which genes are expressed and which are repressed at a given time. If the identical nuclei from a mitosis were distributed into cells with identical cytoplasms, they would govern the development of identical

cell types. If, however, cell division were asymmetrical in some respect, the different cytoplasmic environments of the two nuclei could call forth the expression of different genetic information from each and result in cellular differentiation (Miller, 1985; Miller and Greany, 1976). This was clearly shown by Miller (1987) and Miller and Greany (1976). Exposure of spores commencing germination to two and a half percent methanol inhibited nuclear movement but permitted mitosis and cell division to occur. These spores underwent symmetrical division into two cells of equal size neither of which differentiated into a rhizoid. Instead both cells divided and form "twin" prothalli joined at the base.

The polarity of spore germination in some fern species, while not being affected by gravity, may be oriented by the light stimulus (Verdoorn, 1938). Spores subjected to bright unilateral illumination showed the division wall constantly placed so that the prothallial cell faced towards the light and the rhizoidal cell away from it. The operative factor in eliciting this response is believed to be the difference in light intensity on the two sides of the spore rather than the direction of the light rays (Gantt and Arnott, 1965; Ootaki, 1963). The rhizoidal cell enlarges and grows out, causing the spore wall to separate along the trilete or monolete ridge.

Ultrastrusture of the Primary Rhizoid.

During the partitioning of the rhizoid and prothallial cell, virtually none of the remaining storage protein granules are incorporated into the rhizoid cell. The rhizoid cell is rich in cytoplasmic RNA (Gantt and Arnott, 1965).

Vacuolation of the rhizoid commences during rhizoid emergence and the extension of the vacuoles as the rhizoid elongates affects the distribution of the cytoplasmic organelles, with the greatest organelle concentration occurring at the tip and around the nucleus. Sections through the extreme tip of the rhizoid reveal several small vacuoles and Golgi bodies. An area approximately ten micrometres posterior to the tip appears more vacuolate. The endoplasmic

reticulum (ER) in this area is well developed and runs parallel to the cell wall. Plastids are present but Golgi bodies are less numerous than in the anterior region (Dyer and Cran, 1976).

The cytoplasm of the region about sixty micrometres posterior to the tip and of much of the remainder of the mature rhizoid, has a coarse appearance suggestive of degeneration. The plastids and other organelles also appear to be degenerative, their internal membranes often being considerably swollen (Dyer and Cran, 1976).

The nucleus of the mature rhizoid is elliptical to spindle shaped and occupies a position about half way along the rhizoid, having migrated from its initial basal position (Dyer and Cran, 1976).

Mitochondria are numerous in the rhizoidal cell and resemble those found in higher plants. The cisternae are plate-like with no preferential orientation. Osmiophilic droplets may occasionally be present between the cisternae (Cran, 1979; Gantt and Arnott, 1965).

Golgi bodies are also common and appear to become very active at the commencement of rhizoidal growth. The Golgi bodies consist of a stacks of approximately five flat cisternae from the edges of which vesicles are formed (Cran, 1979). The rapid extension of the rhizoid necessitates a fast synthesis of cell wall material. Vesicles, near the wall of the rhizoid tip and in the cytoplasm, have the same size as the larger Golgi vesicles that are in close proximity to the Golgi stacks. These vesicles are very likely budded-off from the Golgi bodies. Golgi vesicles have also been implicated in wall formation in root hair cells and in cell plate formation (Bell, 1970; Gantt and Arnott, 1965).

The cell wall of the rhizoid differs from that of the prothallial cell in that it is not composed of regularly orientated microfibrils but rather of randomly arranged microfibrils having a loose texture (Smith, 1979). The rhizoid wall consists of two layers. The inner one is electron

transparent, containing, in young rhizoids, a few vesicles and tubules. In fully elongate cells a random array of four nanometre fibrils can be detected. Within this array, dark areas up to sixty nanometres in diameter are present against which the fibrils are often appressed. The outer layer is denser and tangential sections through this layer showed it to be composed of dense masses separated by matrix (Dyer and Cran, 1976).

Gametophytic rhizoids, although having no photosynthetic capacity, have the main functions of anchorage and absorption (Cran, 1979).

One Dimensional Prothallial Development.

The prothallus of the homosporous ferns follows a definite pattern of development leading ultimately to the characteristic adult form. Spore germination results in a uniseriate, elongated germ filament composed of chlorophyllous cells and bearing one or more rhizoids at the basal end. All cell divisions are by cross walls perpendicular to the long axis of the filament (Miller, 1980; Nayar and Kaur, 1971; Davis, 1969; Whittier, 1965).

Growth patterns in plants are usually classified into two categories, tip growth and overall growth (growth throughout the whole cell). Tip growth is mainly found in linearly-growing cells such as pollen tubes, root hairs, fern and moss protonemal cells and fungal hyphae, while overall growth is typified by the cells of higher plants (Doonan and Duckett, 1988; Murata *et al*, 1987).

The basic growth pattern of the protonema of polypodiaceous ferns is tip/apical growth; by elongation and cell division which usually only takes place in the apical cell. These two processes give rise to straight protonema consisting of linearly arranged cells (Ito, 1969). The growth zone is limited to between zero and twenty micrometres from the tip, the same region which shows phototropic and polartropic responses. Both apical growth and the tropic responses

are believed to be under the control of phytochrome, which appears to be located in the plasma membrane, and a blue light-absorbing pigment (Furuya, 1985; Wada and Staehelin, 1981) (discussed later). Since elongation does not occur in the daughter cell basal to the division plane, the cell length of this cell is fixed by the position where cell division occurs in the parent apical cell (Ito, 1969; Ootaki, 1968).

Apical growth implies the local formation of cellulose or other fibrillar wall constituents and local exocytosis of secretory dictyosome vesicles by which pectins and other matrix substances are incorporated into the wall (Devonan and Duckett, 1988; Schnepf, 1986).

a) Ultrastructure of the filamentous prothallus

In the prothallial cell the centrally located nucleus is closely surrounded by numerous lipid bodies, chloroplasts, some mitochondria, small vesicles and scattered Golgi bodies (Wada and O'Brien, 1975). Closely associated with the lipid bodies are tubules of the ER, some of which are continuous with the nuclear membrane (Gantt and Arnott, 1965).

The nucleus is of a fine granular consistency, usually possessing one spherical nucleolus. The apical pole of the nucleus always shows some type of invagination. The invagination contains microtubules or microtubule-like structures. Occasionally these microtubules have been traced through serial sections and have been shown to penetrate ten to fifteen micrometres into the apical cytoplasm from the tip of the nucleus. These microtubules are believed to play a role in both the movement of the nucleus as well as the maintenance of the spindle shape of the nucleus (Wada and O'Brien, 1975).

Mitochondria and Golgi bodies are located rather uniformly throughout the prothallial cell but are not as numerous as in the rhizoid. The matrix surrounding the organelles consists

predominantly of tubules of ER and small granules, presumably ribosomes. Large vacuoles occur at the basal end of the prothallial cell (Gantt and Arnott, 1965).

Chloroplasts are abundant in the prothallial cell. They are surrounded by a double unit membrane and the photosynthetic lamellae are of varied lengths, either single or aggregated into grana. Starch grains within the chloroplast are numerous (Gantt and Arnott, 1965).

Although the chloroplasts vary in size according to the stage of division and the age of the cell, two types of chloroplasts have been distinguished by their shape. The tip of the prothallial cell is rich in cigar-shaped chloroplasts which give the impression that they may be anchored by one end within a thin, moderately electron-dense region of the ground substance that lies close to the cell membrane. Mitochondria often lie in such close proximity to the one end of the chloroplast that they also give the impression of being attached to the plastid envelope by the cytoplasmic ground substance. The other chloroplasts are smaller and rounder (Cran, 1979; Wada and O'Brien, 1975).

All the cells of the germ tube are interconnected by plasmodesmata. The plasmodesmatal canal is lined by a membrane which is continuous with the plasma membrane (Cran, 1979).

The cell wall of the protonemata contain eight nanometre thick, randomly orientated fibrils. In a rapidly growing protonemata the P-face of the plasma membrane contains both randomly distributed particles and distinct particle rosettes. The rosettes consist of six eight to nine nanometre particles in a ring-like configuration and have an outer diameter of twenty-four nanometres. They closely resemble the particle rosettes seen on the P-face of the plasma membranes of green algae and higher plants, which have been implicated in the synthesis of cellulose fibrils. As would be expected with the polar synthesis of cellulose, within the twenty micrometre region from the tip of the protonemata, the region of maximal cell wall growth

and expansion and thus cellulose-fibril synthesis, the greatest density of rosettes is observed in *Adiantum* L. They are almost lacking in the more basal parts of the cell (Schnepf, 1986; Wada and Staehelin, 1981).

In the protonemal wall two layers are usually distinguishable by differences in density and orientation of microfibrils (Smith, 1979).

The surface of the protonemal wall is completely covered by a multi-layered lipid coat, probably consisting of cuticular waxes. The functional importance of this layer is believed to be for reducing the loss of water vapour from the surface of the gametophytes. The rhizoid seems to lack this type of coat (Wada and Staehelin, 1981).

b) Polarity in the filamentous prothallus

The expansion of the cells of the germ filament at ninety to one hundred and twenty degrees to the rhizoid axis is believed to reflect a cytoplasmic gradient inherited from the spore mother cell (Dyer and King, 1979).

The presence of apical dominance and therefore polarity in growth in ferns was shown by Ootaki and Furuya (1969) in *Pteris vittata* L. On the removal of the original apical cell a branch was produced by at least one of the remaining cells in all of the treated protonemata, irrespective of how many basal cells were cut off together with the apical one. Whenever the apical cell was preserved, it continued normal growth and there was no branching in any of the basal cells attached to it. This behaviour was the same as in the intact protonemata which continued normal apical growth without branching from basal cells.

When the basal cells regained the ability to grow and divide after surgical removal of the original apical cell, several physiological and morphological changes occurred in these cells.

One of the earliest visible changes was the increase in volume of the nucleus and nucleolus. The size and shape of the chloroplasts was also observed to change during the re-establishment of apical dominance (Ootaki and Furuya, 1969).

There exist two alternative hypotheses concerning the possible mechanism of apical dominance, namely that the meristem either attracts all substances needed for its development from the basal cells or that it produces an inhibitor which stops the growth of the basal cells. It is uncertain which of these hypotheses operates but it can be concluded that the presence or absence of the original apical cell is the most important factor in controlling the apical dominance during the axial development of *Pteris* L. protonemata (Ootaki and Furuya, 1969).

Interrelated with tip growth, cell organelles are polarly distributed and in some cases even have different structures in different areas of the cell. Perhaps the most striking polar structure in cells with tip growth is the clear cap or tip body. It consists mainly of an apical accumulation of exocytotic Golgi vesicles, together with ER elements. Dictyosomes, ER, mitochondria and sometimes lipid bodies each represent the subapical zone, whereas plastids, if present, follow more basally. The proximal part of a tip growing cell is usually occupied by a vacuole (Schnepf, 1986).

The Role of Microtubules in the Polar Organization of the Organelles and in the Maintenance of Shape.

The cylindrical morphology of the apical cell, the constant maintenance of this shape throughout division cycles, the position of the nucleus and the asymmetric distribution of organelles such as chloroplasts and the establishment of polarity during spore germination are fundamental features of tip-growing organisms and are reflected in the distribution of microtubules in the apical cell (Doonan and Duckett, 1988; Doonan *et al*, 1985).

Microtubules are probably common to most, if not all eukaryotic organisms for they exhibit similarities of function and structure (i.e. the microtubules have an outer diameter of twenty four nanometres, an inner diameter of fifteen nanometres and each microtubule consists of thirteen subunits (Doonan *et al*, 1983 (quoted in Doonan and Duckett, 1988))) which transcend phylogenetic barriers (Mizuno *et al*, 1985; Powell *et al*, 1980).

Microtubules participate as morphogenetic tools in two basic processes by which the plants develop their characteristic forms: (a) production of new cells in specific sites and with specific initial shapes by partitioning of parent cells, and (b) further shaping of the progeny during their expansion and differentiation.

In respect of point (a) microtubules are present in the mitotic spindle, where they develop in the absence of centrioles. Immediately before the division cycle they are deposited as a transitory preprophase band which in its positioning predicts the site and plane of further cytokinesis. At telophase another microtubular system contributes to the organization of the phragmoplast, which contains the new partitioning wall or cell plate (Wick *et al*, 1981).

In respect of point (b), there are many instances of congruence between the orientation of microtubules in the cell during interphase and the orientation of currently deposited microfibrils of cell wall material. For instance in various unicellular and multicellular plants cortical microtubules in the cell are known to be arranged parallel to the orientation of newly deposited cellulose microfibrils and perpendicular to the direction of cell expansion (Sakaguchi *et al*, 1988). The inference is that the cell exerts geometrical control over the expansion by setting up specifically orientated microtubule arrangements. These cytoplasmic "tracks" (microtubules) guide the movement of the cellulose synthesizing complexes along the plasma membrane, thereby regulating the mechanical properties (Wick *et al*, 1981) and in turn, the yielding behaviour of the wall against turgor pressure during growth. Random microfibril orientation is

associated with a mechanically isotropic wall and possession of this arrangement leads to the growth of spherical cells (Miller and Stephani, 1971).

The maintenance of a filamentous one-dimensional protonema therefore probably requires a predominantly transverse orientation of microfibrils. Irradiation with red or far-red light is believed to impose a stricter transverse orientation of cell wall microfibrils and therefore strengthens the wall against lateral expansion, producing filaments with narrow diameters (Stetler and Demaggio, 1972; Miller and Stephani, 1971).

Until recently the only available method for studying the various categories of microtubular arrays was electron microscopy. Immunofluorescence methods, whereby the principal elements of the cytoskeleton, not to mention their associated components, can be investigated in whole cells in three dimensions, would be an ideal alternative to electron microscopy. Immunofluorescence studies of plants have lagged far behind those on animals because of technical difficulties inherent in dealing with walled cells i.e. the cell wall, made up of a polysaccharide and protein complex with a pore size of three to five nanometres, impedes antibody penetration. Reports of successful application of immunofluorescence to plant microtubules are therefore confined to a few where the cell wall presents no such barrier (Doonan and Duckett, 1988; Powell *et al*, 1980) e.g. naked gametes, protoplasts derived from algae, moss protonemata and higher plant cells grown in suspension culture (Wick *et al*, 1981). With the development of a range of procedures for making plant materials permeable to antibodies, while at the same time preserving the integrity of the cell contents, such as the use of non-ionic detergents, specially buffered fixatives and a variety of wall degrading enzymes, it is hoped that immunofluorescence microscopy will be of more use (Doonan and Duckett, 1988).

The polar distribution of vesicles, Golgi bodies and cell expansion found by Schmiedel and Schnepf (1980) (quoted in Doonan and Duckett, 1988) in the apical dome of *Funaria* L.

protonemata a[illegible]dicative of a directional transport system. Such a characteristic distribution of the organelles points to the existence of scaffold force-generating systems that are capable of integrating cellular organisation. However, electron microscopy has failed to reveal a cytoskeletal system within the apical domes of a variety of tip-growing cells. Thus the possible role of microtubules in tip-growth has remained a vexed question, although it is well documented that microtubule perturbing agents cause dramatic growth abnormalities in protonemata (Doonan and Duckett, 1988).

However, the use of microtubule stabilising buffers during fixation combined with indirect immunofluorescence (Doonan *et al*, 1985) clearly demonstrates the presence of microtubules within the apical dome of *Physcomitrella* L. caulonematta. The same technique has also revealed microtubules extending to the tips of root hairs, pollen tubes and fungal hyphae (Doonan and Duckett, 1988).

A study performed by Murata *et al* (1987) using immunofluorescence microscopy distinguished two populations of microtubules within the cortical cytoplasm of *Adiantum capillus veneris* L. protonemal cells growing under red light. The one set was aligned parallel to the long axis of the cell, the other set circumferentially around the cell at the region of the dome except for the extreme apex - none are closer than five micrometres from the tip of the apical dome (Wada *et al*, 1990; Stockwell and Miller, 1974; Stetler and DeMaggio, 1972). A circular array of microtubules suggested by Wada and O'Brien (1975) from thin electron microscope sections correspond to these immunofluorescent observations. Stockwell and Miller (1974) found the microtubules to be orientated in an axial direction in the cylindrical part of the protonemata of *Dryopteris filix-mas* (L) Schott. which were kept filamentous under red light, whereas it was random in plants produced under blue light.

Stetler and Demaggio (1972) found that in filamentous plants grown under red light the orientation of microtubules was not uniform throughout the apical cell. Fifteen micrometres from the apex the microtubules were randomly orientated (this region of the cell has the potential for elongation, and growth), but ten to fifteen micrometres from the tip the microtubule arrangement was longitudinal or axial and no random orientation was observed below twenty micrometres from the tip (Stetler and DeMaggio, 1972). The cylindrical shape of the cell could therefore be maintained by a decreased gradient of synthesis and plasticity of the wall and an increased resistance to turgor pressure behind the tip region. Where no expansion occurs in the filament, axially orientated microtubules were found.

The orientation of microtubules in the bulbous apical cell of gametophytes from blue light differs from that noted in filaments of plants grown in red light i.e. the arrangement of microtubules is random throughout the apical cell and apical swelling occurs. These results suggest that the newly-synthesized cell wall which has a random arrangement of microfibrils expands more transversely than does the cell wall which has a transverse arrangement of microfibrils (Wada *et al*, 1990; Stetler and DeMaggio, 1972).

This pattern of microtubule arrangement has also been found in other tip growing organisms. For example, immunofluorescence microscopy has shown that the moss caulonemata contain microtubules that run in a more or less axial direction. They undulate throughout the cytoplasm, forming a three dimensional meshwork that envelops the nucleus and lipids. In the moss, however, the microtubules invariably fill the apical dome of caulonemata forming a focus at or near the apex of the dome (Doonan *et al*, 1985).

The finding of the circular array of microtubules in protonemal cells lead to the hypothesis that the mechanism of regulation of cell diameter and the resulting growth direction of this tip growing cell may be similar to that in overall growth as seen in higher plant cells. The circular

cytoskeletal structure may control the diameter of the cylindrical cell through the orientated deposition of microfibrils that is regulated by the circular microtubules (Wada *et al*, 1990), and consequently the direction of cell growth (perpendicular to the long axis) may be determined by the physical restriction of microfibrils. The location of this structure at the basal part of the apical dome is consistent with this hypothesis because the growth activity in fern protonemal cells is restricted to the apical hemisphere of the apex.

Microtubules in the rhizoid cell were found to run parallel with the long axis in all parts of the cell, consistent with the electron microscope observations made by Dyer and Cran (1976) of *Dryopteris* Adans. rhizoids. This absence of a circular array of microtubules in the apical dome of the rhizoid wall indicates that the above mentioned hypothesis cannot be applied to the rhizoid. The involvement of an alternative mechanism to keep the cell diameter constant and to maintain the tip growth in the rhizoid cell is required. The concept of the existence of different mechanisms in the regulation of tip growth is supported by the different actions of anti-microtubulin drugs on the tip growing cells. Colchicine treatments induce spherical expansion of apices in fern protonemal cells, however, rhizoids are unaffected by such drugs (Dyer and Cran, 1976).

Although the images may be very striking, the shortcoming of immunofluorescence techniques should not be overlooked. Preparative procedures for visualizing the cytoskeleton are extremely destructive and may thus provide misleading information. Ideally, the immunological probes, which allow visualisation of specific cytoskeletal components by optical microscopy, should also be used in conjunction with gold labelling to extend the observations to the electron microscope level. The latter option is particularly inviting since fixation protocols are now available that preserve both antigenicity and the more labile cytoskeletal domains not previously seen in electron micrographs.

Physiological Differences Between Rhizoid and Protonemal Cells.

Physiological differences between rhizoid and protonemal cells become apparent at approximately the time that elongation of the rhizoid begins. When fully developed, rhizoids and protonemal cells differ in wall structure and staining properties, permeability, uptake of vital stains, osmotic equivalent, behaviour on plasmolysis and distribution of phosphatases.

Most obviously, the protonemal cells are photosynthetic whereas the rhizoids are non-photosynthetic cells which must obtain their energy source and their metabolites required for synthesis of new cell components from the protonema (Smith,1979).

As already mentioned the rhizoid wall is composed of randomly arranged microfibrils while those of the protonemal cells form orientated arrays. Besides these differences in structure, the rhizoid and protonemal cell walls have different staining properties. Rhizoid walls stain purple with toluidine blue but the thallus cell wall shows no reaction unless the cells are damaged. It has been suggested (Crotty, 1967 (quoted in Smith,1979)) that the staining of the rhizoid wall is due to free negative groups of the wall, and that in the thallus cell wall such groups are absent or blocked in some way.

Verifying this, Smith (1979) showed that the rhizoid wall of *Polypodium vulgare* L. contains two types of components which stain with toluidine blue, one staining metachromatically (purple), the other staining orthochromatically (blue). Rhizoid walls contain a high concentration of free negative groups and evidence suggests that these are carboxyl groups. In the protonema, apart from some free carboxyl groups in the pectin fraction of the wall of the basal cell, the carboxyl groups of the walls are esterified and stain with toluidine blue only after saponification.

Because of the presence in it of free carboxyl groups the rhizoid wall exhibits cation exchange properties. It may, therefore, be supposed that it will selectively bind cations, so making them

available in high concentrations near the plasma membrane for uptake into the rhizoid (Smith, 1979).

Development of a Two Dimensional Prothallus.

An abrupt change in the plane of cell division usually occurs when the germ filament becomes two to ten cells long - at this point interstitial divisions are initiated (Dyer and King, 1979). There are basically seven types of prothallial development in homosporous ferns i.e. the *Osmunda*-type, *Marattia*-type, *Adiantum*-type, *Drynaria*-type, *Ceratopteris*-type, *Kaulinia*-type and *Aspidium*-type.

In the *Adiantum*-type development the terminal cell of the germ filament divides by a wall oblique to the long axis of the filament, and this is followed by another division by a wall at right angles to it. Thus, a transverse row of three daughter cells is formed, of which the middle one is wedge-shaped and acts as a meristematic cell (*Fig 3: 8-10*). All cell divisions in the meristematic cell are by walls parallel to the oblique walls, each being perpendicular to the one preceding it. The daughter cells expand and by repeated transverse and longitudinal divisions, form an expanded one-cell-thick obovate prothallial plate (*Fig 3: 13*). The apical cell remains for some time, but is eventually replaced by a group of initial cells (Whittier, 1965). The apex of the thallus at the region of the meristematic cell becomes notched and later cordate (*Fig 3: 13,14*). A midrib is formed by cells behind the meristem in the median plane of the thallus, and a symmetrical cordate prothallus results with a median midrib and semicircular lateral wings (*Fig 3; 17*). The sex organs and rhizoids are usually restricted to the midrib on its ventral surface (Nayar and Kaur, 1971).

The *Drynaria*-type of development differs from the *Adiantum*-type in that the establishment of an apical meristematic cell is markedly delayed. A broad spatulate prothallial plate is formed (*Fig 3: 11,18,19*) by division of the anterior cells of the germ filament. A meristematic cell is

formed in one of the anterior marginal cells of the prothallial plate when it is five to ten cells broad (*Fig 3: 20*). Further growth is as in the *Adiantum*-type and results in a cordate thallus (Nayar and Kaur, 1971). In the *Kaulinia*-type a prothallial plate develops as in the *Drynaria*-type, but with this type (*Fig 3: 26*) the cells of the entire anterior region of the thallus become meristematic. The thallus is ribbon-shaped (*Fig 3: 27*), with its anterior end smoothly rounded (not notched). Branches may develop (Nayar and Kaur, 1971). The early stages of the *Ceratopteris*-type of development are also similar to the *Drynaria*-type, and a broad prothallial plate is formed. No meristematic cell is differentiated, but meristematic activity gradually becomes restricted to a group of marginal cells on one side of the plate, away from the apical region (*Fig 3: 21*). This lateral meristematic region differentiates into a pluricellular meristem and forms a notch (*Fig 3: 22,23*). A midrib is formed behind the meristem. The young thallus is thus cordate but asymmetrical with one wing larger than the other (*Fig 3: 24*) (Nayar and Kaur, 1971).

In the *Osmunda*-type of development, characteristic of the Osmundaceae, a plate of four cells is formed (*Fig 3: 1,2*). By repeated divisions in all the four cells and expansion of daughter cells, a nearly circular broad prothallial plate is formed (*Fig 3: 3*). A meristematic cell is established in a marginal cell towards the middle of the group (*Fig 3: 4*). The meristematic cell grows faster than the others, and the thallus becomes asymmetrical (*Fig 3: 5*). The young thallus becomes notched at the meristematic region, and develops into a symmetrically cordate structure as in the *Adiantum*-type (Nayar and Kaur, 1971). As in the *Osmunda*-type, the *Marattia*-type of development results in a symmetrical cordate adult prothallus. This type of development is restricted to ferns in which spore germination is of the amorphous-type (*Fig 3: 6,7*) (Nayar and Kaur, 1971).

The *Aspidium*-type differs from all others. The terminal cell of the germ filament produces a unicellular papillate hair crowning it and becomes languid (*Fig 3: 29*), taking little part in

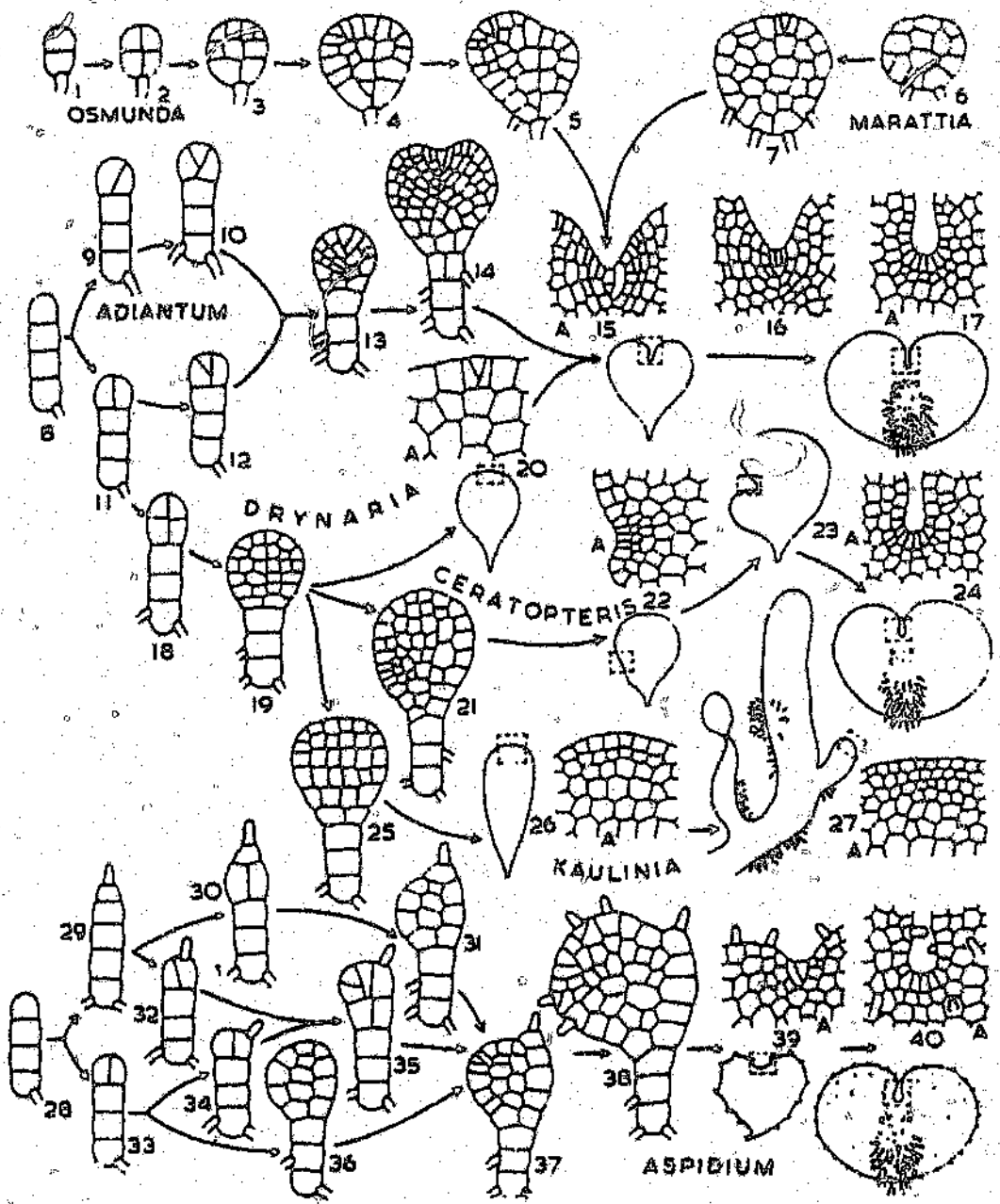


Figure 3: A schematic representation of the type of prothallial development which occurs in homosporous ferns. The arrows indicate successive stages in each type of prothallial development as follows: numbers 1, 2, 3, 4, 5, 15, 16, 17-*Osmunda*-type; numbers 6, 7, 15, 16, 17-*Marattia*-type; numbers 8, 9, 10 (or 8, 11, 12), 13, 14, 15, 16, 17-*Adiantum*-type; numbers 8, 11, 18, 19, 20, 15, 16, 17-*Drynaria*-type; numbers 8, 11, 18, 19, 21, 22, 23, 24-*Ceratopteris*-type; numbers 8, 11, 18, 19, 25, 26, 27-*Kaulinia*-type; numbers 28, 29, 30, 31, (or 28, 29, 32, 35 or 28, 33, 34, 35 or 28, 33, 36), 37, 38, 39, 40-*Aspidium*-type (adapted from Nayar and Kaur, 1971).

development. A broad plate with one side broader than the other forms behind the languorous anterior region. A meristematic cell is differentiated in one of the marginal cells on the more expanded side of the plate (*Fig 3: 37*). Marginal unicellular hairs are produced continually. Development of the thallus after the establishment of a meristematic cell is as in the *Adiantum*-type (*Fig 3: 38,39*). The young prothalli are lopsided, but as the thalli grow, the asymmetry is lost and the adult thallus is symmetrically cordate (*Fig 3: 40*) (Nayar and Kaur, 1971). Variations on this type of prothallial development are described by Nayar and Kaur (1971).

Two main hypotheses have been proposed to account for the transition from the filamentous prothallial phase to the two dimensional phase of growth. The first hypothesis has been stated most fully by DeMaggio and Raghaven (1973) (quoted in Smith,1979). The transition is considered to be an obligate photomorphogenesis, i.e. filamentous growth only changes to two dimensional growth in the light, with blue light being most effective in eliciting the transition (Davis, 1968). Blue light is believed to activate a set of genes which produce mRNA coding for proteins necessary for two dimensional growth (Miller, 1980; Bopp, 1967; Steeves *et al*, 1955).

In the second hypothesis the primary factor controlling the transition is held to be the balance between cell division and cell elongation (Smith, 1979; Sobota and Partanen, 1966). A low rate of division permits the cells to elongate, and divisions are orientated to produce a septate filament. Increasing the rate of division is thought to decrease the potential for cell elongation, and division in two or more planes is promoted. The hypothesis states that the transition will occur when the dividing cell is wider than it is long since the plane of minimal area, in which the new cell wall will form, will be longitudinal (Stetler and DeMaggio, 1972; Sobota and Partanen, 1966). A variety of factors could be implicated in controlling this pattern of development and there is considerable evidence that chemical substances which promote cell elongation or inhibit cell division will prevent two dimensional growth and enhance filament formation (Miller, 1980; Stetler and DeMaggio, 1972; Steeves *et al*, 1955).

Miller and Stephani (1971) have proposed that certain wavelengths of light mediate changes in the orientation of cytoplasmic microtubules which, in turn, are responsible for changes in the orientation of cellulose microfibrils of the cell wall. They suggest that these changes in the patterns of cellulose deposition are ultimately responsible for the form of the fern gametophytes produced under various light conditions.

The attractiveness of the latter hypothesis is that it focuses on the cell wall as its primary site for changes leading to the development of different cell forms. However, none of these hypotheses are adequate to explain how the transition is controlled in a constant environment in which all factors are favourable from before spore germination (Smith, 1979).

Although developmental changes in the protonema have received scant attention, sufficient information is available to indicate that even under constant conditions changes occur which may be preliminary steps leading to the two dimensional transition (Smith, 1979).

Hotta and Oswa (1958) described an increase in the relative protein content associated with the transition to two dimensional development in *Dryopteris erythrosora* Sim. (Davis, 1968; Bell and Zafer, 1961). This increase is predominantly associated with the chloroplasts (Bopp, 1967). It is probable that photomorphogenetic light reactions can determine into which metabolic pathways the primary products of photosynthesis are channelled. A photoreactive system which depends on blue light apparently directs this flow of metabolites primarily in the direction of protein synthesis. With the aid of this channel many more products of photosynthesis can be used for protein synthesis in blue than in red light (Mohr, 1961-64).

Hotta(1960) (quoted in Smith, 1979) reported a marked increase in RNA and protein per unit dry weight and per plant at the time of the transition to two dimensional growth. It is possible

that the transition requires the synthesis of a new species of RNA and protein which should and is detected as qualitative differences between filamentous and two dimensional plants.

Sexual Reproduction.

Once mature the heart-shaped gametophytes produce sex organs.

Development of antheridia.

The antheridia are the first sex organs to develop on fern gametophytes, appearing three to four weeks after germination in *Ceratopteris thalictroides* Sim. They are generally formed on the basal portion of the chordate prothallus and often arise from superficial cells along the margin (DeMaggio, 1977; Pal and Pal, 1963). The cells of the lower epidermis which give rise to the antheridia in the Filicineae are first distinguishable from the other epidermal cells by their denser cytoplasm and slightly smaller chloroplasts. As the cells round up and divide to form the young antheridium, there is a gradual absorption of starch, the chloroplasts decrease in size, becoming spindle-shaped. The cells of the antheridium contain a sparse cytoplasm, small green plastids and vacuoles full of phenolic compounds (Verdoorn, 1938).

The beginning of antheridial development is noted by the protrusion of a superficial cell which divides periclinally separating a hemispherical antheridial initial from the basal cell. The antheridial initial subsequently divides anticlinally several times before another periclinal division produces a primary spermatogenous cell. At an early stage this cell is surrounded by sterile tissue, which, through continued divisions, forms the antheridium. The primary spermatogenous cell divides in a regular fashion and soon a mass of cells with large, distinct nuclei is formed within the antheridium. As the sperm cells continue to develop the nuclei begin to flatten and the protoplasmic material in the cells begins to condense (Sheffield and Bell, 1987; DeMaggio 1977)

The induction of antheridium formation in fern prothalli is controlled by endogenously formed inductive substances, known as antheridogens. The antheridogen has been chemically characterised and found to be structurally related to gibberellin (Takeno and Furuya, 1980; Näf, 1969; Bopp, 1967). The mechanism of their action is however not yet well understood.

The spermatogenous cells possess a large centrally located nucleus with a prominent nucleolus and a very dense cytoplasm containing vesicles and organelles. During differentiation and maturation of the spermatocytes the blepharoplast is produced. This structure ultimately gives rise to the flagellar apparatus. The blepharoplast consists of tubular structures or cylinders. Microtubules radiate from it in all directions and appear to terminate close to its perimeter (DeMaggio, 1977).

The mature spermatozoids are symmetrical cells roughly five micrometres long by five micrometres wide, bound by a plasma membrane and having the form of a sinistrally coiled helix. The spermatozoid consists principally of the multilayered structure (MLS - derived from the blepharoplast), mitochondria, nucleus and a ribbon of aligned microtubules running the length of the gamete. The flagella occupy the anterior portion of the cell. It is generally believed that the shape of the helical gamete is maintained by the backbone or microtubular ribbon which acts as an elastic cytoskeleton (Walker, 1985; Demaggio, 1977).

Development of archegonia.

In nature the archegonia usually arise on the shaded or lower surface of the prothallus behind the apical notch or meristematic region of the heart-shaped gametophyte. They develop from superficial cells which, because of their dense cytoplasm, can be identified as archegonial initials. The initial cell first produces a row of three cells from which the archegonium and its contents will be derived. During development the outer wall divides and ultimately produces the protruding neck of the archegonium (Sheffield and Bell, 1987). The innermost cell divides next,

producing derivatives which form the basal cells of the jacket or venter. Finally, the central or primary cell in the row of three produces the neck canal cells, the ventral canal cell and the egg. Growth of the primary cell is considered to be the first significant event in oogenesis (DeMaggio, 1977).

The primary cell when first formed is box-like in appearance but as differentiation progresses it enlarges often to five times its original size. The young cell possesses a large nucleus with irregularly shaped nucleoli and an organelle-rich cytoplasm containing numerous ribosomes and polysomes (Bell, 1970). As the cell enlarges many changes take place in the cytoplasm. Plastids begin to differentiate and grana and starch are lost. The cytoplasm becomes very dense and the large vacuoles disappear. Their disappearance is accompanied by the appearance of a large number of small vesicles, the origin of which is unknown (DeMaggio, 1977;Bell and Mühlethaler, 1964).

As the cell continues to enlarge most of the cytoplasm remains at the base of the archegonial cell. Subsequent division of the primary cell produces two cells of unequal size - a small neck canal cell and a larger central cell. The neck canal cell is situated towards the archegonial neck and has only a small amount of cytoplasm while the central cell inherits much of the cytoplasmic mass of the primary cell. The central cell undergoes an unequal division producing a highly cytoplasmic egg, occupying the base of the archegonium, and next to it in the archegonial neck, the ventral canal. There is a continuity of cytoplasm from the primary cell to the central cell and eventually to the egg (DeMaggio,1977).

Once formed the egg undergoes a series of significant cytological and biochemical changes as it matures. Initial observations of the structural changes which take place during egg maturation depicted the degeneration of plastids and mitochondria and the evagination of the nucleus into the cytoplasm (DeMaggio, 1977).

The mature egg of *Pteridium aquilinum* (L.) Kuhn is surrounded by an acetolysis-resistant membrane. The strong affinity of the egg membrane for Sudan black B and the necessity for benzpyrene staining to render it fluorescent, led Cave and Bell (1974) to conclude that it consists predominantly of lipid, possibly in the polymerised form.

The egg membrane of *Pteridium* Scop. appears coincidentally with the discharge of vesicles from the cytoplasm and since these vesicles are believed to arise from the autophagy of at least some of the mitochondria and plastids an efflux of lipid materials to the surface of the egg is not unexpected, and could provide the precursors of the osmiophilic membrane.

The obliteration of plasmodesmata which were present during early stages of oogenesis seems to further isolate the egg from neighbouring cells. Therefore, despite the fact that the young embryo is in intimate physical contact with the gametophyte during this period, results of experiments suggest that there may be an incomplete metabolic union between the two generations i.e. autoradiography showed that radioactive amino acids fed to the gametophyte concentrate at the boundary of the embryo with little or no radioactivity being found in the embryo. On general principles of permeability, it would be expected that other molecules of similar or larger size would also fail to cross this boundary. There is a strong possibility, therefore, that the sporophytic embryo draws only on "simpler" molecules from the gametophyte and that the sporophyte has a metabolic autonomy *ab initio* (Bell, 1961).

This osmiophilic layer is therefore thought to provide an effective barrier to the entry of complex metabolites into the egg during the final stages of its differentiation and conversely the layer might also reduce the tendency of metabolites to diffuse out of the zygote and young embryo (DeMaggio, 1977; Cave and Bell, 1974; Bell and Mühlethaler, 1962; Bell, 1961).

As maturation proceeds, the ventral canal cell, situated adjacent to the egg, degenerates and the neck canal cell also breaks down. These events occur at the time when a considerable increase of cytoplasmic DNA can be measured in the egg (Sheffield and Bell, 1987).

Fertilization.

Dehiscence of antheridia generally occurs in ferns only minutes after the gametophytes have been in contact with the water. Water entering the antheridia raises the internal pressures and causes the antheridial walls to swell. A short time later the opercular cells open (Nayar and Kaur, 1971). Discharge of the coiled sperm follows quickly. In the Polypodiaceae the male gametes are discharged from the antheridium coiled within a membrane and the spermatozoids become active only as this mucilaginous sheath surrounding them is dissolved and they begin a rapid rotary motion. Swimming sperm appear to be attracted to the archegonia by the mucilaginous protoplasmic material released during archegonial opening.

Ordinarily, within one hour after coming into contact with water, mature archegonia begin to open. Movement of globules in the neck canal and the swelling of the terminal cell indicate that the archegonium is about to open. Shortly thereafter, a small amount of mucilaginous material is exuded between the neck canal cells of the apical tier. The hole at the tip of the archegonial neck enlarges as the neck cells begin to separate and once this hole is large enough, the bulk of the mucilaginous material from the neck canal discharges forcefully. Once the neck canal contents have been eliminated the terminal cells of the neck begin to part in an orderly fashion with the rows of neck cells reflexing toward the gametophyte surface (Whittier and Peterson, 1980; DeMaggio, 1977). The expelled material, remnants of the neck and ventral canal cells, usually remains in the vicinity of the opened archegonia but does not obstruct the passage to the egg.

Some attention has been given to the identification of the substances that diffuse from the archegonia during dehiscence and are responsible for the chemotactic response of ferns. It has been shown that C4 acids, especially malic acid and its salts, have the ability to attract sperm of many ferns. This substance, or some related compound, is considered to be excreted by cells of the axial row establishing a gradient toward which the sperm swim (DeMaggio, 1977).

The actual mechanism by which the sperm penetrates the egg is still in question. Penetration is attributed to the helical motion of the sperm which is directly controlled by the flexing movement of the microtubule band (Sheffield and Bell, 1987). Egg penetration is, therefore, a purely physical process (Sheffield and Bell, 1987; Miller, 1985; Myles *et al*, 1978).

The sperm entering the neck of the archegonium are elongated and do not retain their characteristic helical form. Accompanying the change in shape, the sperm shows signs of disorganisation. Nuclei and flagella appear disrupted and the chromatin condenses along one side into a dense rod (Myles *et al*. 1978; DeMaggio, 1977). The microtubule band is observed to separate from the nucleus. No cilia are observed attached to the spermatozoid, but in the cytoplasm on the side toward the archegonium neck are found definite hair-like structures. One is inclined to interpret them as cilia thrown off as the spermatozoid makes its way toward the egg.

Entrance of the sperm also causes physical disruption of the cytoplasm and this is thought to be responsible for rapid structural changes in the egg. Since no special fertilization membrane is found to block entrance of additional sperm into the egg cytoplasm, prevention of polyspermy is attributed to changes taking place in the egg cytoplasm after sperm entry (DeMaggio, 1977). At the time when the spermatozoid is found touching the egg nucleus, the cytoplasm at the base of the egg appeared conspicuously vacuolate. After penetration, the spermatozoid nucleus

undergoes a slow process of expansion in the nucleus of the egg, until the two constituents become indistinguishable (Verdoorn, 1938).

Once fertilization has been completed the young embryo becomes turgid and fills the archegoniate venter. At this time, the cytoplasm is unevenly distributed and the embryo appears highly vacuolate. During the next few days the cytoplasmic contents become more evenly distributed, the embryo increases in size and the jacket cells or calyptra enclosing the egg begins periclinal division (Sheffield and Bell, 1987; DeMaggio, 1977).

Development of the sporophyte.

The first organ to be clearly distinguished is the foot. The foot serves as a haustorium and is considered to be the absorbing organ for the young embryo and its cells often intrude between cells of the parent gametophyte (Verdoorn, 1938). In cultured *Lycopodium appressum* L. the foot cells and contiguous gametophytic cells develop extensive wall ingrowths, and they are considered to be transfer cells. Transfer cells in the foot of young sporophytes and in adjacent gametophytic cells have numerous mitochondria, plastids with variable amounts of starch and elongated, narrow wall ingrowths forming a labyrinthine wall-membrane apparatus. Transfer cells in older interfaces have thickened wall ingrowths, few mitochondria, plastids with numerous plastoglobuli and little starch and a large central vacuole. Plasmodesmata do not develop between cells of sporophyte and gametophyte generations and these are, therefore, isolated symplastically during all stages of sporophyte development (Peterson and Whittier, 1991).

While the foot is in the process of forming the leaf initials appear in a position lateral and distal to the foot. Stem and root initials are formed within one to two days after the differentiation of leaf initials. As the shoot develops procambial tissue differentiates acropetally into it. About thirty days after fertilization the root begins to break through the calyptra and approximately

two days later the primary leaf straightens and grows over the prostrate prothallus. The young sporophyte is now prepared to begin an independent existence (Sheffield and Bell, 1987; DeMaggio, 1977).

The free-living, relatively large, sporophyte characteristic of pteridophytes was generally considered to have been made possible by an evolution of adequate tissues for the upward and downward conduction of materials. All pteridophytes have two general types of conducting tissues, the xylem and the phloem. The chief function of the former is the upward conduction of water, that of the latter is the upward and downward conduction of foods. Together they constitute the chief elements in the vascular system of the sporophyte. The tracheid is the fundamental element of the xylem and is matured from a single embryonic cell. An embryonic cell destined to mature into a tracheid elongates greatly and deposits additional wall material upon limited portions of the inner face of the cell wall. Sooner or later there is a disappearance of the cytoplasm and nucleus. A mature tracheid, therefore, is an elongated dead cell consisting of only a cell wall. The additional wall material is deposited in transverse rings - they are therefore referred to as annular tracheids. Tracheids mature in regions of the stem, root or leaf that are still elongating. The phloem of pteridophytes is composed of sieve tubes and phloem parenchyma (Smith, 1938).

Because sporophytic growth can be obtained directly from gametophytes without gametic fusion, and fully functional gametophytes from sporophytes without reduction of chromosome number or of the amount of DNA in the nuclei it has been suggested that the environments in which the spore (initial cell of the gametophyte generation) and the zygote (initial cell of the sporophyte generation) begin to develop are so different that quite different sets or number of genes are activated in each cell, with striking morphogenetic consequences. If this were true, a spore placed in the situation of a zygote might be expected to yield a sporophyte, and a zygote, removed from the archegonium and germinated as a spore, a gametophyte (Bell, 1970).

Bell and Jayasekera (1959) (quoted in Bell, 1970) cultured isolated archegonia containing fertilized eggs and found in every instance that the zygote yielded a normal sporophyte. The amount of gametophytic tissue surrounding the zygote was so little that the presence of the gametophyte was not considered essential for the zygote to develop into a sporophyte.

DeMaggio and Wetmore (1961) were able to extract very young embryos from archegonia of *Todea* Willd. Using suitably enriched media they found that, whereas old embryos produced normal sporophytes, the young embryos could only produce prothalloid outgrowths which resembled gametophytic rather than sporophytic tissue. DeMaggio and Wetmore (1961) concluded that the cause of this reversion to the gametophytic morphology was the removal of the young embryo from an especially complex nutritive environment provided by the archegonium. This view, though plausible, seems less credible when examined in detail, e.g. there are doubts whether complex molecules can in fact pass from gametophyte to sporophyte due to the presence of the osmiophilic membrane which surrounds the mature eggs of ferns (see p. 32).

If the environment of the mature archegonium is not responsible for the properties of the zygote, two other possibilities are open. One is that the activation of the genes responsible for sporophytic growth is a consequence of fertilization, and the other that the activation occurs before fertilization, most probably during the differentiation of the female gamete (Bell, 1970).

In the ferns, the entire spermatozoid enters the egg. The nucleus of the spermatozoid immediately penetrates the female nucleus, but the motile apparatus (the flagella, basal bodies and fibrils) and mitochondria remain in the cytoplasm and is believed to be digested away. Whatever its fate, the cytoplasm contributed by the spermatozoid is trifling in quantity and specialized in function. The nuclei of spermatozoids consist of strands of DNA closely packed in longitudinal alignment. It is conceivable, but unproven, that the nucleus of the spermatozoid

takes particular histones or other chromosomal proteins, able to reorientate the activities of the genes, into the female nucleus (Bell, 1970).

GENETICS OF THE SEXUALLY REPRODUCING FERNS.

Whilst the history of flowering plant cytology extends back to the last century that of the ferns may be said to start in 1950 with the publication of Manton's book "Problems of Cytology and Evolution in the Pteridophyta". Prior to this date only a few sporadic chromosome counts were reliable, the whole topic having been inhibited by the high chromosome numbers encountered and the difficulties of obtaining preparations of sufficient quality to ensure complete accuracy (Walker, 1979).

In general, fern chromosomes do not show morphological detail with the clarity that is visible in flowering plants and even the exact position of the centromere in some cases may not be absolutely clear.

The sporangium of leptosporangiate ferns follows a precise and regular ontogeny; the sporangium is initiated from a single initial cell, and after the sporangium is well developed, a single archesporial cell develops and undergoes mitosis to form two cells, thence another mitosis and four cells, another mitosis and eight cells, and a final mitosis resulting in sixteen spore mother cells. When these spore mother cell undergo meiosis sixty four spores result which have half the chromosome number of the sporophyte (Klekowski, 1979).

One of the most obvious characteristics of the chromosomes of ferns is the high number involved and Klekowski (1973) (quoted in Walker, 1979) calculated that the mean gametic number of homosporous pteridophytes was about fifty four. Such high numbers originate from two sources, namely a high initial base number and superimposed polyploidy. The base numbers range from twenty two to sixty nine in all but a handful of ferns (Walker, 1979).

The high base number is assumed to have been derived from lower ones in the past by a process that Chiarugi (1960) (quoted in Walker, 1979) termed paleopolyploidy - an ancient multiplication of an "ancestral" base number. The inference that low numbers did once exist receives support from two sources - the persistence of low numbers in certain groups and from karyological studies. The low numbers occur commonly in heterosporous pteridophytes, for example *Sellaginella* P. Beauv. has n = 7, 8, 9 and 10; *Isoetes* L. has n = 10 and 11 and the heterosporous aquatic *Salvinia natans* Desv. has n = 9. Low numbers are also known in a few homosporous ferns, such as *Hymenophyllum peltatum* (Poir.) Desv. with n = 11 and *H. tunbrigense* (L.) J. E. Sm. n = 13 (Walker 1979).

Evidence has also come from karyotype analysis by the Japanese school. Tatuno and Kawakami (1969) (quoted in Walker 1979) suggested that the chromosomes of *Asplenium* L. and *Camptosorus* L. could be grouped into six types, each of which could be divided into two groups. From this they deduced that the ancestral base number of these genera is twelve. Similarly for *Osmunda* L. and *Planasium* L. of the Osmundaceae with present day n = 22, Tatuno and Yashiua (1966'1967) (quoted in Walker, 1979) concluded that present-day numbers were in reality tetraploids on n = 11. This latter conclusion has been supported by Klekowski (1970) (quoted in Walker, 1979) who states that the genetics of *Osmunda* indicates duplicated loci. In all these cases the supposed ancestral numbers gave rise to present day base numbers by straight polyploidy.

In addition to high base numbers, polyploidy is one of the outstanding features of ferns, occurring at a conservative estimate in fifty to sixty percent of all the cytotypes. However, whilst there is an overall high incidence of polyploidy it demonstrates few obvious rules in that it is not uniformly distributed among the various genera and families, nor is it especially a characteristic of more primitive or more advanced groups, nor of habitat, etc. Thus it is common in Marattiaceaea, Ophioglossaceae and Hymenophyllaceae, whilst apparently being

totally absent in Osmundaceae and Cyatheaceae and of only sporadic occurrence in Dicksoniaceae and Gleicheniaceae. In virtually all other families of any size polyploidy is frequent. Again, it appears to matter little whether the base number is high or low as regards the incidence of polyploidy. For example, at the lower end of the scale all the members of Osmundaceae (n = 22) are diploid in the wild and the same is true towards the other end of the scale with *Cyathea* Smith on a base number of sixty nine. An extreme case is *Ophioglossum* L. in which, despite a presumed base number of about one hundred and twenty, numerous polyploids are known up to approximately the decaploid level. Many of the larger genera show differences in both the percentage of polyploidy and the levels reached. The genera *Pteris* L. and *Adiantum* L. have similar total percentages of polyploidy but only approximately three percent of the species are at a higher level than tetraploid in *Pteris*, whilst the figure has risen to fourteen percent in *Adiantum*.

Polyploid production in the ferns has been observed in a number of well documented cases, primarily in the genus *Asplenium* L. Wagner and Whitmire (1957) (quoted in Walker, 1979) reported that a wild diploid hybrid, *A.* x *ebenoides* (*A. platyphyllum* (L.) Oakes x *A. rhizophyllum* Hieron.), with complete failure of meiotic pairing, spontaneously gave rise in cultivation to a tetraploid plant which paired its chromosomes and produced crops of fully viable spores. The diploid *A. viride* L. hybridizes with both diploid subspecies of *A. trichomanes* L., giving rise to *A.* x *protoadulterinum* when crossed with subsp. *A. trichomanes* and *A.* x *adulteriniforme* with subsp. *inexpectans*. Both hybrids show seventy two univalents at meiosis and both can give rise to fertile tetraploid progeny by chromosome doubling (Walker, 1979).

Close examination of the *Asplenium* hybrids showed that although some sporangia contained abortive material, others contained a variable number of well filled spores or an admixture of viable and abortive spores. During meiosis, cells at metaphase I are abundant, whilst little evidence was found of later stages. The evidence is that meiosis frequently aborts after

prolonged metaphase and is followed by restitution of the nucleus, effectively circumventing a reduction in chromosome number and producing diploid instead of haploid spores (Walker, 1979).

That chromosome doubling during sporogenesis is a relatively common occurrence is further attested to by the anomalous results that are sometimes obtained by workers synthesizing hybrids. A number of hybrids have been produced which are morphologically "correct" but which have an enhanced chromosome number over that expected from the parentage. Thus occasionally a tetraploid hybrid will be produced from a tetraploid x diploid cross instead of the expected triploid. The probable explanation is that the diploid has produced a few diploid spores which have germinated to give rise to diploid prothalli and gametes. Fertilization has then occurred involving gametes from the "new" diploid prothalli and those of the normal diploid prothalli of the tetraploid species. This has been recorded in *Dryopteris* Adans, *Pteris* and in *Phyllitis* Hill.(Walker, 1979).

In most instances polyploidy inv(s hybridization followed by chromosome doubling. Chromosome doubling is normally the second stage in the production of a new polyploid but there may be a considerable time lag between hybridization and polyploid establishment. The most conspicuous genetic feature of homosporous pteridophytes is that they produce homothallic prothalli which can bear both male and female sex organs at the same time and may become completely homozygous by a single act of self-fertilization. A single spore that has successfully doubled its chromosome number is potentially capable of germination and producing a prothallus which can, by self-fertiliztion, give rise to a polyploid sporophyte, i.e. a single act. This aspect will be dealt with in more detail later. A heterosporous plant on the other hand must have a combination of relatively rare events occurring together for a polyploid to be established. There must be at least two separate acts of creation occurring more or less simultaneously,i.e. romosome doubling in the formation of the megaspore and also of the

microspore. Furthermore, since separate male and female gametophytes are produced there is no possibility of self-fertilization and the polyploid egg cell must be fertilized by a polyploid spermatozoid from another gametophyte. Again, the chances are that the polyploid egg cell would be fertilized by the much commoner normal spermatozoid rather than by the relatively rare polyploid sperm. In brief, the heterosporous plant in order to establish polyploidy must meet very rigorous conditions both in time and in space, whereas the homosporous plant has to fulfil much less demanding conditions. Hence the much greater incidence of polyploidy in homosporous ferns might be expected *a priori*.

As already mentioned, homosporous ferns have sporangia which form spores having the capacity to give rise to gametophytes that are exosporic and hermaphroditic i.e. forming antheridia and archegonia on the same gametophyte. The maximum inbreeding that is possible in such a system is the fusion of egg and spermatozoid which have originated from the same gametophyte and, since this gametophyte is the product of mitotic divisions of a spore genotype, all the gametes produced by this gametophyte have the same genotype. Thus the union of two gametes produced by the same gametophyte results in a zygote that is completely homozygous. Therefore, homosporous ferns have the capacity to form fully homozygous zygotes in one generation of selfing. This life cycle characterises the majority of extant ferns (Klekowski, 1979).

Because of the complexities which are possible in fern reproductive biology, very specific terminology has been derived to describe the various levels of crossing and selfing that are possible in these organisms. Selfing can be defined as the fusion of gametophytes derived from the same parental sporophyte, but due to the possibility of forming hermaphroditic gametophytes two levels of selfing are possible, i.e. the fusion of gametes produced by the same gametophyte and the fusion of gametes produced by sib gametophytes. The probability of a genotype being homozygous for a given gene where selfing is a result of the fusion of gametes

formed by sib gametophytes is half whereas the probability that a gene locus is homozygous where the gametes originate from the same gametophyte is one (Klekowski, 1979).

An additional point is that the zygote resulting from the fusion of gametes coming from the same gametophyte is homozygous at every gene locus whereas the probability of a completely homozygous zygote occurring where the gametes come from sib gametophytes in $(½)^n$ where n is the number of heterozygous loci in the parental sporophyte genotype. The following terminology is used to describe the genetics of these organisms:-

1) Intragametophytic selfing - the fusion of sperm and egg from the same gametophyte. Normally this results in a completely homozygous zygote.
2) Intergametophytic selfing - the fusion of sperm and egg from different gametophytes with both gametophytes being sibs i.e. originating from the same parental sporophyte. This is analogous to the self fertilization of a seed plant.
3) Intergametophytic crossing - the fusion of sperm and egg from different gametophytes with each gametophyte originating from a different parental sporophyte. This is analogous to cross-fertilization of a seed plant.
4) Intergametophytic mating - the fusion of sperm and egg from different gametophytes with the origin of the gametophytes not specified (Klekowski, 1979).

APOGAMY.

Although the life cycle described is the most common one for ferns, it is not the only one in existence. Some ferns have abandoned sexual reproduction, completing their life cycles by the production of spores without a reduction in chromosome number (diplospory) and by prothalli which produce sporophytes without gametic fusion (apogamy) - the whole process being repetitive and obligate (Walker, 1985; Whittier, 1965).

History and Origin of Apogamy.

The term apogamy was proposed by DeBary in 1878, (quoted in Steil, 1951) following Farlow's discovery (1874) that in *Pteris cretica* L., under artificial culture, the sporophyte is developed from the gametophyte with the suppression of the sexual act (Dyer and King, 1979; Steil, 1951; Steil, 1939; Yamanouchi, 1908).

The natural occurrence of apogamy has been described for many species of fern and occurs in most fern families (White, 1979; Whittier and Steeves, 1960). De Barry (1878) carried out an extensive study on a number of ferns in the Polypodiaceae, in which he described apogamy in *Aspidium filix-mas cristatum* L. and *A. falcatum* L. Sadebeck in the following year reported apogamy in *Todea africana* Willd and later apogamy was found in *Osmunda regalis* L., and *Ceratopteris* A. Brongn. (Lietgeb, 1885); *Todea rivularis* Willd., *T. pellucida* Willd. (Stange, 1887); *Doodya caudata* L. (Stange, 1887); *Trichomanes alatum* v. d. Bosch (Bower, 1888); *Selaginella rupestris* Sprenger (Lyon, 1904); *Trichomanes krausii* Willd., *Pellaea flavens* (Willd.) Hook., *P. nivea* (Willd.) Hook., *P. tenera* (Willd.) Hook., *Notholaena eckloniana* Kunze, *N. sinuata* Kunze, *N. marlothii* Hieron., *Gymnogramme farinifera* Desv. (Woronin, 1907) and in many others (Yamanouchi, 1908).

The origins of apogamous species are still a matter for conjecture. The cytological evidence strongly suggests a hybrid origin e.g. out of a total of one hundred and twenty five apogamous species in the floras of New Zealand, Jamaica, Britain, Ceylon, North America, Malaya and Japan no less than eighty nine are triploid (Walker, 1979).

An analysis of agamosporous species, make it clear that several different and essentially independent genetical factors have been brought together in order to produce a working system - factors affecting both sporophytes and gametophytes. It seems likely that the factors have been assembled piecemeal by repeated hybridizations between different genotypes, having different

components of the system. Once assembled, the entire system is inherited as a unit and in every cross made between a plant of the Döpp-Manton type (to be discussed later) and sexual species, the offspring are apomictic (Walker, 1979).

Development of the Apogamous Sporophyte.

Apogamy in the Pteridophyta is of two kinds, obligate and facultative.

Obligate apogamy

Obligate apogamy describes the kind of cycle in which the sporophyte is produced regularly from the gametophyte without sexual fusion. The life cycle of the obligatorily apogamous ferns is almost identical to that of their sexual relatives. In *Dryopteris bornei* Kuntze, for example, the young gametophytes produce antheridia and the spermatozoids are capable of fertilizing the eggs of related sexual species. Archegonia, if they are produced, do not function - this has been attributed to "poor development" or "necrosis" of the egg cells. In the place of archegonia, a second meristem arises within the somatic cells of the gametophyte located behind the apical meristem and yields a sporophyte directly (El Dosouky *et al*, 1990; Sheffield and Bell, 1987; Bell, 1979; White, 1979). *Pellaea atropurpurea* (Sw.) Link is another apogamous fern whose unreduced gametophytes do not make functional archegonia or eggs but do make functional antheridia or sperm (El Dousky *et al*, 1990).

No specific cell of the gametophyte shows a change before the apogamous development of the sporophyte, which might correspond to the differentiation of the egg. However, once maturity is reached, mitoses take place in rapid succession in the vicinity of the sinus (notch). Mitosis continues and cell plates are laid down parallel or oblique to the surface of the prothallium, the ultimate result being the initiation of a thick cushion region (Verdoorn, 1938; Yamanouchi, 1908). A three-sided apical cell develops or a group of distinctly marked embryonic cells appears in this cushion region from which the sporophyte arises (Bopp, 1967; Whittier, 1962;

Duncan, 1941). In some fern species such as *Heilanthes tomentosa* and *H. alabamensis*, this "embryonic" region appears as a small tan region behind the apical notch. The colour change is believed to result from a modification of chloroplasts in the meristematic cells of this region (Whittier, 1965). In some instances, when the apogamous sporophyte is about to make its appearance, a small projection is produced in the apical notch of the prothallium.

Usually the first evidence of apogamy is the appearance of glandular hairs such as are common on typical embryos and young leaves. The hairs appear on the elevated region of the prothallus which differentiates early from the rest of the prothallus and which consists of cells resembling meristematic tissue. From this a leaf unfolds, and later the stem initial and other leaves and roots appear (Lawton, 1936).

Shoot and leaf apical organization occur early with the roots arising endogenously at a later stage (White, 1979) i.e. the apogamous sporophyte lacks primary roots - adventitious roots arise from the side of the stem.

The apogamous nature of the buds and sporophytes is demonstrated by the direct connection of the sporophytic tissue and the absence of a foot-like region (White, 1979; Whittier, 1976).

In Yamanouchi's (1908) description of the origin of the embryo in *Nephrodium (Dryopteris) molle* Bak. the embryo was believed to originate from a single haploid cell on the ventral side of the prothallium a short distance back from the apical notch. The initial cell was distinguished by its larger size, larger nucleus and denser contents (Steil, 1951; Duncan, 1941). In the apogamous ferns investigated by Duncan (1943) (quoted in Steil, 1951), the origin of the embryo could not be traced to a single cell, but rather to a number of cells of the gametophyte.

According to Duncan (1943) (quoted in Steil, 1951), no evidence of the apogamous embryo can be observed until the prothallium posterior to the apical notch consists of four layers of cells. The upper two layers remain distinctly gametophytic and therefore are not involved in the formation of the embryo. From the other two layers the whole embryo develops with the apical cell of the stem being produced from the fourth layer - a segment of this apical cell functions as the apical cell of the leaf. In the third layer the apical cell of the root is differentiated (Steil, 1951).

So far as they have been investigated, the meristem which gives rise to the apogamous ferns is at all times in normal continuity with the surrounding gametophytic tissue. There is also no evidence that these cells are isolated from the gametophyte by any kind of boundary resembling that around the egg and zygote, nor that their cytology is in any way different from that of normal meristematic cells (Bell, 1979). The initiation of sporophytic outgrowths does not involve necrosis of adjacent prothallial cells (Sheffield and Bell, 1987).

All the indications are, therefore, that the spore itself possesses the capacity of ultimately producing a sporophyte and no further transformations are necessary. The transition from gametophyte to sporophyte cannot, however, be a consequence of the unreduced chromosome number (relative to the parent) since in sexual species stable gametophytic morphology is compatible with diploid nuclei. Hence, the conclusion seems inescapable that whatever endows the spore with the capacity to produce a sporophyte is independent of the number of sets of chromosomes and is likely to be cytoplasmic (Bell, 1979).

The apogamous outgrowths are believed to originate in one of three ways:-

1) by the continued growth and division of jacket cells of abnormal antheridia whose interior cells do not form antherozoids;

2) by the continued growth and division of the basal cells and superficial cells about the venters of the archegonia; and

3) by the elongation outward and the subsequent division of the upper surface of the gametophyte.

Apogamous outgrowths produced by methods one and two abort early in development (Steil, 1939). Therefore only the last named produces mature apogamous sporophytes.

The shoot apical meristem of the apogamous sporophyte is covered by a number of leaf bases and the young leaf primordia are enclosed by stipules or leaf sheaths of the next older leaves. Leaves on apogamous and sexually produced sporophytes are similar with long, thin petioles and blades that constitute only a small fraction of the leaf length. However, the blade of the first leaf from the apogamous sporophyte is three-lobed instead of showing the two-lobed condition typical of sexually produced sporophytes (Whittier, 1976; Duncan, 1941).

The conversion from gametophyte to sporophyte in apogamous ferns is associated with an exponential increase in proteins (Bopp,1967).

Facultative apogamy.

Induced or facultative apogamy is the development of apogamous sporophytes from gametophytes which have functional sex organs and normally give rise to sporophytes by syngamy under other circumstances (Whittier and Steeves, 1960; Duncan, 1941). Facultative apogamy can be induced in a number of ways, usually involving manipulation of the environment (Whittier, 1962).

Depriving gametophytes of water (thus preventing the opening of the archegonia and release of the spermatozoids) is often effective (Sheffield and Bell, 1987; Walker, 1985; Steil, 1951; Duncan, 1941).

High levels of illumination promote apogamy and high concentrations of glucose, sucrose, fructose and maltose led to an increased frequency of apogamous outgrowths (Elmore and Whittier, 1975). For example, in the fern *Pteridium aquilinum* (L.) Kuhn, which has sexual prothalli, apogamous sporophytes can be induced by glucose (two and a half percent is maximally effective) or other sugars added to the medium (Bo[illegible]967). This tends to indicate that a high nutritional status favours apogamous gametophytic growth (Loyal and Cho[illegible]ra, 1977; Munroe and Sussex, 1968; Whittier, 1964; Bristow, 1962).

The way in which a high concentration of sugar in the nutrient medium causes a thalloid gametophyte to give rise directly to a vascular sporophyte has been investigated. One possible explanation might be that the induced apogamy is in some way a response to the increased osmotic pressure presented by the higher concentration of glucose in the medium (Whittier and Steeves, 1960). This view is supported by the observation that the addition of mannitol, a nutritionally inert substance for *Pteridium* Scop. gametophytes, can partially replace the sugar needed for the induction of apogamy. Mannitol increases the osmotic concentration of the nutrient medium without increasing the respiratory state (Whittier, 1975).

An alternative hypothesis to explain the role of sugar in the induction of apogamy is that it functions as a respiratory substrate, thereby increasing the energy available to the organism (Whittier and Steeves, 1960; Whittier, 1962). This is supported by the observation that since a threshold amount of sucrose or glucose is needed for apogamy, it would appear that an increase in respiratory substrate above that produced by photosynthesis is required for apogamous sporophyte formation (Whittier and Steeves, 1962).

Ethylene has also been shown to promote apogamy in ferns. How ethylene works is still largely unknown but it is thought to channel the energy supplied by sucrose toward the formation of

apogamous buds whereas in the absence of ethylene the energy from the sugar would be used to produce more gametophytic tissue (Elmore and Whittier,1975).

Elmore and Whittier (1975) recognised two stages in the induction of facultative apogamy in *Pteridium*. The first stage required both high carbohydrate levels and ethylene, the second only carbohydrates (White, 1979; Bopp, 1967).

Naphthylacetic acid (NAA) (optimal concentration two milligrams per millilitre), indoleacetic acid (IAA) and Gibberellic acid (GA) promote the formation of apogamous sporophytes, especially when applied together with four percent sucrose. The formation of apogamous structures therefore depends upon a sufficient supply of carbohydrates and other organic substrates. Carbohydrates are not regarded to be direct triggers of differentiation; they permit to a greater extent the activity of those genes which are necessary for the differentiation of the sporophytes (Treanor and Whittier, 1969; Bopp, 1967).

White (1979) noted that the occurrence of apogamy was greater where a larger number of gametophytes forming the colony in the culture dish of a specific size were present. This is probably due to the larger amounts of ethylene released by the gametophytes into the culture solution.

In summary therefore, the apogamous embryo differs from the sexual one in four principal ways:-

1) The apogamous embryo is intimately connected with the prothallium in such a way that one cannot decide where one begins and the other ends;
2) There is no foot or equivalent organ formed;
3) The vascular bundle of the sporophyte is in direct connection with vessels which lie in the prothallium; and

4) The order of evolution is different, a leaf arising first and becoming tolerably well developed before the root and afterward the stem make their appearance (Steil, 1951; Yamanouchi, 1908).

The Genetics of Apogamy.

The absence of fertilization during apogamous reproduction results in the reproduction of the same genome, generation after generation and in the gametophyte and sporophyte having the same chromosome number. In obligate apogamy sporogenesis occurs regularly, but sporophyte and gametophyte retain the same chromosome numbers (Sheffield and Bell, 1987; Sheffield and Attree, 1983).

For the origin of the apogamous embryo of two ferns, Farmer and Digby (1907) (quoted in Steil, 1951) described an unusual history. They described the origin of the embryo from a single diploid cell produced by the fusions of the prothallial cells and their nuclei. The fusion of these cells, in the cushion of the gametophyte was described as a "substitution" fertilization. Numerous studies on the embryo of apogamy have been made, but so far no one has confirmed the observations of Farmer and Digby (Steil, 1951; Steil, 1939). Steil (1939) stated that their observations were based on artifacts of fixation.

Approximately one hundred and forty cryotypes have been confirmed as agamosporous, amounting to about ten percent of those ferns for which the breeding system is known, and are confined to only eighteen out of the three hundred and fifty to four hundred genera commonly recognised. Nearly all follow the Döpp-Manton scheme.

During sporogenesis in sexually reproducing ferns the initial archeosporial cell in the developing sporangium divides four times by mitosis resulting in sixteen spore mother cells. Meiosis occurs, giving rise to sixty four spores with half the original chromosome number. In apogamous ferns

modifications of either mitosis or meiosis occur, resulting in the production of viable spores but which have the sporophytic instead of the reduced chromosome number. This system has been designated the Döpp-Manton scheme (Walker, 1979).

In the Döpp-Manton system two types of cytological behaviour are shown by the majority of the sporangia. In one case the archesporial cell divides four times by mitosis to give rise to sixteen spore mother cells and then meiosis occurs, just as in the a sexual species. However, meiosis is irregular and consequently only abortive spores are produced. In the other type of behaviour shown, a compensation mechanism occurs, resulting in regular meiosis and the production of viable spores. Here three mitotic divisions give rise to eight spore mother cells. A further mitotic division starts normally, the chromosomes move to the equator and divide but there is no separation towards the poles and restitution nuclei are formed. As there is no cytoplasmic division involved during this mitosis the result is the formation of eight spore mother cells, each with double the original chromosome number. Each chromosome now has an exact partner available with which it pairs and the following meiosis is perfectly regular with only bivalents formed. As a result thirty two diplospores are produced which have the same chromosome number and genotype as the sporophyte (Walker, 1985; Walker, 1979; Whittier, 1965).

Braithwaite (quoted in Walker, 1979) described another type of agamospory. Here the archesporial cell undergoes four normal mitotic divisions to produce sixteen spore mother cells. At the first meiotic division the chromosomes move towards the equator without pairing. The cytoplasm fails to divide and restitution nuclei are formed with the result that there are still sixteen cells without any alteration in chromosome content. The second meiotic system is normal, giving rise to up to thirty two viable diplospores arranged in diads and having the original sporophytic chromosome number (Walker, 1985; Walker, 1979). Again, prothalli are

produced which form sporophytes apogamously. Thus, although the outcome is the same as in the Döpp-Manton scheme, the cytological means by which it occurs is different.

In Döpp-Manton apomicts, the sporangia which form the diplospores regularly produce the full complement of thirty two spores. However, in the Braithewaite type, this figure frequently falls short of this, anything from twenty three (or less) up to thirty two being produced. This shortfall is due to irregular cytoplasmic cleavage and results in the presence of small abortive fragments intermixed with the viable spores or of mishappen ones (Walker, 1985). In the Döpp-Manton apomicts, because agamospory is inherited as a dominant character, the fertilization of sexual species by the functional spermatozoids of the apomicts can lead to the build up of large agamosporous complexes.

All the Braithwaite apomicts are members of species complexes and differ widely in their systematic position. This contrasts with the Döpp-Manton apomicts which are predominantly concentrated in Adiantaceae and Dryopteridaceae with only a few isolated examples found outside these families (Walker, 1985).

Advantages of Apogamy.

The occurrence of apogamy is unrelated to taxa (Smith, 1979). This has lead workers to conclude that this method of reproduction has arisen independently many times because of the advantages it provides over sexual reproduction.

The spores of the apogamous fern are generally larger with the greater volume being occupied by cytoplasm. As a consequence of this extra cytoplasm, apogamous gametophytes mature faster than those of their sexual relatives and apogamous sporophytes appear well before archegonia in related sexual species grown under similar conditions (Whittier, 1970).

Many apogamous species are able to thrive in relatively xeric habitats eg *Pellaea* Link and *Notholaena* R. Br. This is possible because the sexual species are dependent upon the availability of free water for reproduction while the apogamous ones are not (White, 1979; Nayar and Kaur, 1971; Whittier, 1965; Foster and Gifford, 1959).

This factor, together with the larger spore size, has lead to the idea that apogamy is an adaptation to dry habitats. However, this cannot be held as a generalization since many of the genera with numerous apogamous species are not xeric plants (Walker, 1979).

Other advantages of apogamy mentioned by Anthony (1983) are:-

1) By eliminating sexual reproduction apogamous plants can retain all the accumulated genes which favour survival in a particular habitat e.g. in the cheilanthoid and adiantoid ferns the adaptation would be to xeric environments and
2) because antheridia are found in most apogamous gametophytes studied, such ferns are still able to function as male parents to sexual forms and thus form hybrids which may exhibit hybrid vigour. As mentioned above, genetic control of apogamy has been found to be dominant - this dominance would be effective in perpetuating and increasing the number of apogamous ferns by crosses involving antherozoids from apogamous plants.

FACTORS AFFECTING GAMETOPHYTE DEVELOPMENT.

The development of the fern gametophyte, whether sexual or apogamous, is affected by a number of rather diverse treatments. William (1938) (quoted in Sobota and Partanen, 1966) and Kato (1964) noted that the form of the prothallus is readily influenced by a number of factors including supply of mineral salts, variations in temperature and osmotic pressure and in the intensity and spectral quality of light.

1) Nutrient Media

Gametophytes have been cultured on several different media and it appears that, once germinated, they will grow on any medium containing the macro-nutrients. Deficiencies in macro-nutrient supply affect the development of the gametophytes, e.g. the development of the prothallus may be arrested at the stage of a cell plate without meristem by cultivation in a substratum devoid of nitrates (Verdoorn, 1938). Sufficient micronutrients are usually present in the spores and as impurities in the other organic compounds. However, the rate and even the detailed pattern of development can be affected by the concentration and composition of the medium. The pH of the medium needs to be in the range of four and a half to seven for optimal germination (Dyer, 1979).

2) Population Density

Population density also plays a role in development of the fern gametophyte. An increase in population density leads to an increase in gametophyte length and a delay in the transition to two dimensional growth (densities over one hundred gametophytes per millilitre of medium) (Dyer, 1979; Verdoorn, 1938). The inhibition of development associated with high populations has been attributed to starvation presumably as a result of competition for nutrients. Too low a level of light through mutual shading was also thought to be responsible for the response. Recently studies have shown that a volatile substance is released by the germinating spores which inhibits germination at some stage prior to nuclear division; it also inhibited protonemal cell division but promoted elongation (Smith, 1979; Smith and Rogan, 1970).

Spores germinating in isolation (below one millimetre square on fifteen millilitres of agar (Dyer, 1979)) often gave rise to nodular masses of cells (Smith and Rogan, 1970; Steeves *et al*, 1955).

Density of sowing also has a marked effect on reproductive organ development with maleness tending to predominate in crowded cultures. This is thought to be due to a build up of antheridogens in the surrounding culture medium (Bell, 1979).

3) Colchicine Treatments

The effects of colchicine have been tested on germinating spores of several fern species. The commonest effect of colchicine is hypotrophy of the spore, which swells to form a giant spherical cell and does not undergo any division. This effect can be interpreted in terms of colchicine disruption of microtubules, leading to failure of mitosis and random orientation of cellulose microfibrils in developing wall. The new wall is unpolarized, resulting in the formation of a spherical cell (Dyer, 1979).

4) Growth Substances

a) Auxin

IAA induces rhizoid initiation from the basal cell, so that up to three secondary rhizoids were present in addition to the primary rhizoid. Later development was abnormal: swelling or branching of the rhizoids occurred and protonemal growth was inhibited (Smith, 1979).

b) Gibberellic acid

GA will substitute for light in the induction of spore germination. It was proposed that the gibberellin activates stable mRNA present in the dry spore which code for proteins necessary for early events in germination, including reserve hydrolysis, division of the spore nucleus and initiation of protonemal cell and rhizoid (Smith, 1979).

5) Light

Light is well known to be the most influential of the various environmental factors that control the developmental processes of ferns. In general, red light promotes germination and filamentous elongation of the protonema, perpetuating indefinitely the pattern of one dimensional growth. Blue light promotes active cell division and the conversion to two dimensional prothallia. When a gametophyte cultured under red light is transferred to blue or white light, the rate of elongation is reduced and the apical region of the protonema becomes swollen (Racusen *et al*, 1988; Grill, 1987; Wada and O'Brien, 1975; Stetler and DeMaggio, 1972; Sobota and Partanen, 1967).

Experiments performed by Cooke and Paolillo (1979a) have shown that under all light qualities the actual rate of volume increase within a species is equal, i.e. the cross-sectional area of the filament compensates for the differences in the rates of elongation. Red light therefore promotes elongation rate but the filaments are narrow, while blue light enlarges the cross-sectional area and the increased mitotic rate caused by the blue light enhances the tendency of the elongate cell of the filament to assume an isodiametric shape. Cooke and Paolillo (1979b) believe that this isodiametric shape is the essential precondition for the transition to two dimensional growth.

The photoreceptivity of the fern gametophyte is believed to be due to the involvement of phytochrome and one to two additional photoregulatory pigments (Wada and Kadota, 1989; Wada, 1985; Greany and Miller, 1976).

The effects of continuous red light i.e. the continued elongation of the filament with the clustering of the majority of the cell contents around the nucleus, leaving an almost vacuolate basal region are well documented but, as yet, it is not known how these effects are mediated. Because the effects of the red light treatment can be reversed by far-red light the

phenomenon is thought to be mediated by phytochrome (Wada and Kadota, 1989; Greany and Miller, 1976). The intracellular localization of the phytochrome was studied by Wada and Furuya (1978) using microbeam analysis. The photoreceptive sites for red light were not found to be limited to any particular region of the protonema, but were always located at the cell periphery therefore suggesting that the phytochrome localizes on or close to the plasma membrane. This finding supports the hypothesis that the primary action of phytochrome is by changing the membrane permeability.

Red light induced membrane depolarization has been detected in *Onoclea* Sw. (Wada and Kadota, 1989). The membrane potential was repolarized by far-red light which is typical of a phytochrome mediated response. There is, however, no direct evidence that these electrophysiological responses are involved in the primary action of the following sensory transduction chains leading to photomorphogenic responses.

Originally it was thought that phytochrome mediated the blue response as well. However, while phytochrome does absorb blue light, the efficiency and relative effectiveness are too low to account for the observed blue-light induced phenomena (Cooke and Paolillo, 1979b). Rather, the transition from filamentous to two dimensional growth seems to involve the photoinducible reorientation of the plane of cell division (Wada and Furuya, 1978). It has been proposed that this transition to two dimensional growth involves the blue-light mediated induction/suppression of specific genes that code for proteins necessary to initiate or sustain two dimensional growth (mentioned above).

Using polarized light and /or microbeam irradiation of blue light showed that only the irradiation in the region containing the nucleus could induce cell division (Wada and Kadota, 1989; Wada, 1985; Cooke and Paolillo, 1979b; Wada and Furuya, 1978). However, surrounding the nucleus is a layer of chloroplasts and a thick layer of cytoplasm containing

oil droplets, mitochondria, small vacuoles, endoplasmic reticulum etc. Therefore it is still unknown whether it is the nucleus itself which is the photoreceptive site, or some organelle closely associated with the nucleus.

Changes in membrane permeability are induced by blue light irradiation and are considered by Wada and Kadota (1989) to be an early step in signal transduction leading to blue light mediated responses. Blue light promotes cell division by shortening the G1 phase of the cell cycle. How it does this is still unknown.

At least three different morphogenetic reactions in the early development of *Anemia phyllitidis* Sim are under phytochrome control. In addition to the induction of spore germination and determination of cell shape, red-light irradiation also inhibits antheridium production by antheridogens and gibberellins (Schraudolf, 1987).

It is evident that elementary processes of development in fern gametophytes are greatly influenced by light, and that the processes are controlled antagonistically by phytochrome and one or more blue and near-ultraviolet light absorbing pigments. The interaction between the effects of the two pigments must take place somewhere in their effector systems rather than direct interaction between the pigments, because the intercellular localisation of the pigments is clearly separated inside the cell (Furuya, 1985).

Although a large amount of research has been done on the fern gametophyte, a number of questions still remain unanswered, especially regarding the alternative forms of reproduction such as apogamy, therefore providing scope for this investigation i.e., to restate briefly the aims that were given in full on pages 1 & 2,

1) to establish the origin of the apogamous bud,

2) to determine the delineation of the apogamous embryo and

3) to compare the ontogeny of the apogamous sporophyte with that in sexually reproducing ferns.

CHAPTER 3

MATERIALS AND METHODS

EXPERIMENTAL ORGANISM.

The fern used in this study was identified as *Cheilanthes viridis* (Fonsk.) Swartz var. *glauca* (Sim) Schelpe and Anthony, using herbarium specimens and the identification key in Burrows (1990). Voucher specimens (*Anderson 1 and 2 J*) are housed in the Moss Herbarium, University of the Witwatersrand, Johannesburg.

Mature fronds of *C. viridis* var. *glauca* were collected from the top of a rocky outcrop in the Lovers Rock Pleasure Resort, Magaliesburg in March 1990. The fronds were placed in Petri dishes lined with filter paper and left at room temperature in the dark for one week. This treatment resulted in the mature sporangia drying, splitting open and releasing the mature spores. The released spores were then passed through four to six layers of lens tissue to remove sporangia and leaf fragments. The spores were collected and stored at room temperature in glass vials for use throughout the study.

CULTURE CONDITIONS.

In order to determine which nutrient medium and substrate type would best suit the germination requirements and the subsequent development of the gametophytes of *C. viridis* var. *glauca* a number of trial experiments were performed.

In the first trial experiment one percent agar was added to each of the following nutrient media:- Knops solution (Appendix II) (Loyal and Chopra, 1977; Trivedi and Bajpai, 1977; Nitsch, 1951), Moores medium (Appendix II) (Douglas and Sheffield, 1990; El Dousky *et al*, 1990; Evans and Bozzone, 1977), Mohr's medium (Appendix II) (Grill, 1987; Dyer and Cran,

1976), Knudsons medium (Appendix II) (Whittier, 1981; Steeves *et al*, 1955), Provosoli Enrichment Medium (Appendix II) (Stein, 1973) and Bolds Basal Medium (Appendix II) (Davis, 1969). The pH of the media was adjusted to between five and seven since this range has been found to be optimal for germination and gametophyte development (Dyer, 1979). The media were autoclaved for twenty minutes at 120°C and, under sterile conditions, ten millilitre aliquots poured into sterile Petri dishes with 5cm x 5cm grids drawn on the base (*Fig. 4*). There were three replicate dishes for each of the nutrient media.

Once the agar had set, single spores were placed in each block of the grid using a single bristle brush. The Petri dishes were sealed with masking tape and placed in a growth chamber maintained at 20°C, with a photoperiod of eight hours dark: sixteen hours light and an irradiance of 17μmolm^{-2}s^{-1}. After one week the Petri dishes were removed and the spores examined on an inverted microscope for signs of germination. However, all the Petri dishes showed excessive fungal contamination and very little spore germination. It was thought that the presence of the fungi was affecting the spore germination therefore a second trial experiment was performed.

This trial experiment involved repeating trial experiment one except that this time surface sterilised spores were used in an attempt to decrease fungal contamination. The sterilization technique used was a combination of the methods used by a number of authors including Douglas and Sheffield (1990), El Dousky *et al* (1990), Miller and Wagner, (1987), Minamikawa *et al* (1984) and Haufler and Gastony (1978). Spores were placed in centrifuge tubes containing a 1% Tween solution. The tubes were agitated until all the spores were suspended after which they were centrifuged at 2700rpm for four minutes. The liquid was decanted. Following two cycles of rinsing in sterile distilled water and centrifugation, the spores were suspended in sterile distilled water and filtered onto nucleopore filters using a nucleopore filter apparatus. The

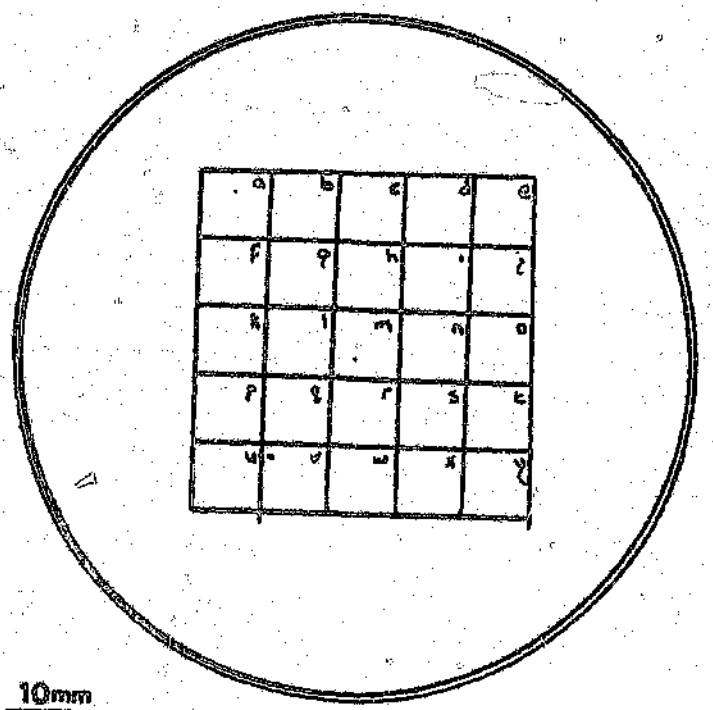


Figure 4:- A diagrammatic representation of the agar plates used to culture gametophytes in the trial experiments.

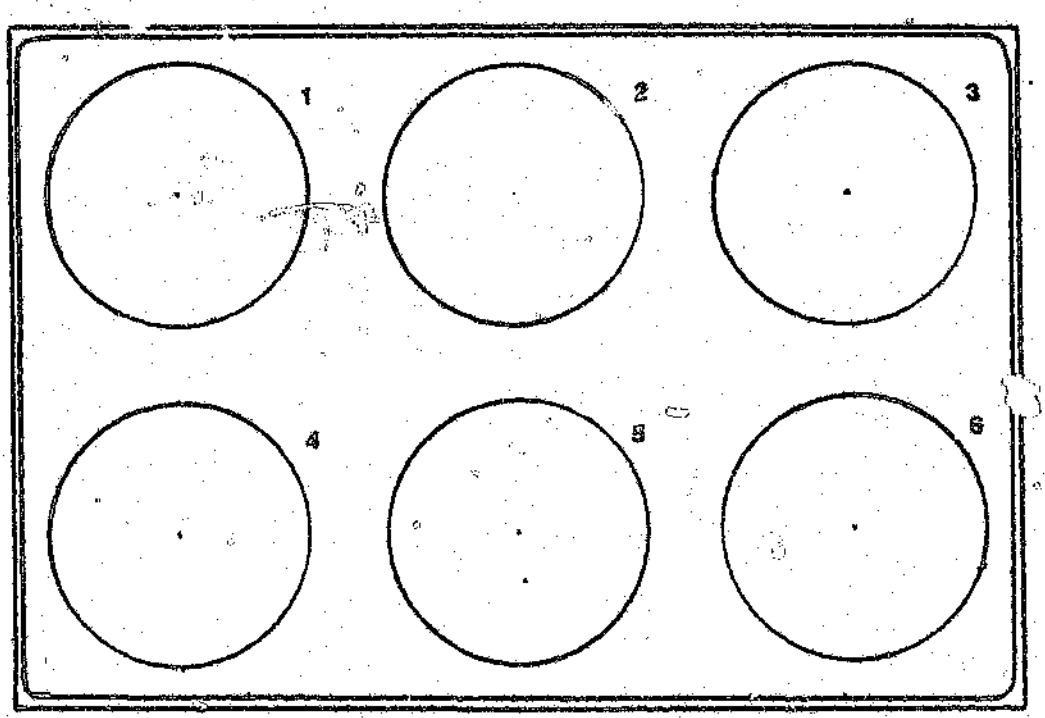


Figure 5:- A diagrammatic representation of a six welled Repli dish.

spores were then sterilized for one, two and a half, five and seven and a half minutes respectively by passing a 20% (v/v) sodium hypochlorite solution (Jik™) through the filter for the allotted time. The spores were then washed thoroughly with sterile distilled water and plated on the various solid media as described for trial experiment one.

After nineteen days in culture it was found that Bolds Basal, Moores and Knops media had the least fungal infection and the highest percentage of germination. However, the highest percentage germination obtained was still relatively low, being forty three percent after one minute sterilization and cultured on Knops medium. Another problem was that the development of the gametophytes was stunted - after thirty days in culture the protonema consisted of only two cells and very few chloroplasts were present in either of these cells. After forty six days two dimensional growth had been initiated but the cells were almost devoid of cell contents. No further development of the protonemata took place.

A third trial experiment was performed in an attempt to increase the percentage germination and to initiate normal gametophyte development. Bolds Basal, Moores and Knops media were used in this trial experiment since they had given the highest percentage germination in the previous trial experiments. The liquid media were autoclaved at 120°C for twenty minutes and, under sterile conditions, three millilitre aliquots were placed into the wells of a Repli dish (*Fig 5*). Unsterilised spores were dusted onto the surface of the liquid media using a paint brush. Four replicates for each medium were used. The Repli dishes were sealed with masking tape and placed under the same culture conditions as before.

When the Repli dishes were examined three to five days later it was found that there was almost one hundred percent germination of the spores on all the media tested. Further development of the gametophytes took place as expected. This was therefore the method of

culture used. Knops medium was chosen out of the three because the initial development of the gametophytes on this medium was most rapid.

For this study, therefore, the gametophytes of *C. viridis* var. *glauca* were cultured on liquid Knops medium in a growth chamber with the following settings:- 20°C, photoperiod of eight hours dark: sixteen hours light and irradience of 17μmolm^{-2}s^{-1}.

LIGHT MICROSCOPE STUDIES.

Fresh Material.

At various stages in the development of the fern gametophyte samples were removed from the Repli dishes and mounted in a drop of nutrient media on slides. These whole mounts were viewed on a Zeiss photomicroscope and the results recorded photographically and diagrammatically.

Fixed Material.

Gametophytes showing the development of the young apogamous bud, the mature apogamous bud and the young sporophyte were embedded in L R White Resin and sectioned using the following procedure:

Samples of gametophytes showing the relevant stages of development were removed from the Repli dishes and fixed for not less than sixteen hours in 6% GTA in phosphate buffer (Appendix II). The fixed gametophytes were then washed three times in phosphate buffer for a total of thirty minutes and dehydrated in a graded alcohol series. The absolute alcohol was replaced with L R White Resin and the gametophytes were left in L R White resin overnight. The specimens were orientated in foil embedding dishes in fresh LR White. The resin was then allowed to polymerise in a 60°C oven for twenty hours. Semi-thin sections (0.5 - 0.7μm) were cut on a Reichert OMU3 microtome. The sections were picked up onto clean slides and dried down onto the slides using a hot plate (80°C). Some of the sections were stained with toluidine

blue (Appendix II) while others were stained as explained in the Histochemical studies section (p 69).

ELECTRON MICROSCOPE STUDIES.

Scanning Electron Microscopy.

The spores of *C. viridis* var. *glauca* were examined using the scanning electron microscope in order to obtain details of the surface ornamentation. Due to the thick exospore, the spores required no processing treatments before being mounted onto five centimetre stubs using "Nappy glue" as the adhesive. The specimens were coated with gold palladium before being viewed on a JSM-840 Scanning Electron Microscope.

Transmission Electron Microscopy.

The spores of *C. viridis* var. *glauca* proved to be more difficult to fix and infiltrate with resin than the gametophytes. For this reason the time periods for the processing of the spores and the gametophytes for transmission electron microscopy (TEM) differ significantly.

The methods employed for the fixation, postfixation, dehydration and resin embedding are based on those of Hayat (1986), Kuehnert and Larson (1983), Lintilhac and Jensen (1974) and Gantt and Arnott (1965).

Processing of fern spores for TEM.

The method described here is one of many which were tried and is the one from which the results shown in this dissertation were obtained.

Fern spores were centrifuged at 2700rpm in a 1% Tween solution for five minutes. After two washes in distilled water and centrifugation, the spores were processed as follows:-

1) The distilled water was decanted and replaced with 6% glutaraldehyde (GTA) made up in 0,2M phosphate buffer (pH 6,9). The test tubes containing the spores and GTA were sealed with Parafilm and left for two weeks at room temperature.

2) Fixation was followed by three phosphate buffer washes over the next three days to remove all traces of the GTA.

3) Post fixation was in 1% osmium tetroxide (OsO_4) made up in 0,2M phosphate buffer for one week and was followed by three phosphate buffer washes, each being for a period of twenty four hours.

4) The dehydration procedure followed is indicated in *Table 1*;

TABLE 1: The dehydration protocol used for spore dehydration.

ALCOHOL (%)	TIME (hrs)
30	4
50	16
70	24
90	24
100	24
100	1 week

5) An extended resin infiltration process was carried out in an attempt to gain adequate infiltration of the Spurr's resin (Appendix II) into the spores. The absolute alcohol was replaced with a mixture of 75% alcohol and 25% Spurr's for four hours. The specimens were then placed in a mixture of 50% alcohol and 50% Spurr's for two days. A mixture of 25% alcohol and 75% Spurr's replaced this solution for two days. The specimens were then placed in 100% Spurr's for a week, in fresh Spurr's for a day and then embedded in fresh Spurr's

in foil embedding dishes. The embedded specimens were polymerised at 70°C for sixteen hours.

Processing of fern gametophytes for TEM.

A similar method, but with greatly reduced time periods, was used for the preparation of the fern gametophytes for TEM observations:-

1) At various stages in their development, gametophytes were removed from the Repli dishes and fixed in 6% GTA in 0,2M phosphate buffer for a minimum of sixteen hours.
2) The fixation was followed by three washes in phosphate buffer over a half hour period.
3) Postfixation was in 2% OsO_4 made up in 0,2M phosphate buffer for two hours.
4) Three washes in phosphate buffer, each for ten minutes, followed the postfixation.
5) Dehydration of the specimens was carried out through the same graded alcohol series used for the spores, but over only an hour and a half period instead of over eleven days.
6) The specimens were infiltrated with Spurr's resin over a period of three days as described in Appendix II.
7) Specimens were embedded in fresh resin in foil embedding dishes and the resin was polymerised for sixteen hours at 70°C.

Sectioning.

Sections of the embedded spores and the various stages of gametophyte development were cut on a Reichert Ultracut E microtome using glass knives made on a LKB 7801 B knife maker. Gold to silver-gold sections, 60 - 90 nm thick, were picked up on to three hundred mesh copper grids. Semi-thin sections, approximately 0.45µm thick, were cut, placed onto glass slides and stained with toluidine blue for light microscope observations.

Staining.

The ultra-thin sections on the grids were stained with uranyl acetate (Appendix II) for twenty five minutes, washed in distilled water and then stained in lead citrate (Appendix II) for twenty minutes. The grids were then washed with 0,02M sodium hydroxide and rinsed in distilled water. These staining times were found to produce adequate contrast in the material.

Staining was carried out in the standard way by floating the grids carrying the sections, section downward, onto individual drops of the appropriate stain on pieces of parafilm. The staining procedure was carried out in Petri dishes to ensure that the grids were kept dust free.

Sections were viewed in a JEM 100S transmission electron microscope operating at 80kV.

HISTOCHEMICAL STUDIES.

Various histochemical procedures were carried out on the gametophyte material which had been embedded in L R White Resin.

Combined Test for Protein and Polysaccharides.

Aniline blue black, Periodic acid-Schiff (PAS) reaction.

Aniline blue black stains proteins a clear blue colour. The chemical basis of the test is not known but the dye is used for the quantification of proteins separated by disc electrophoresis (Fisher, 1968). The PAS reaction imparts a brilliant red colour to all polysaccharides. The basis of this reaction is that the polysaccharide molecule is acted upon by the oxidizing agent, the most commonly used oxidant being periodic acid (HIO_4); this results in the production of aldehydes which are then reacted with leucofuschin, contained in the Schiff reagent, to produce the highly coloured complexes.

The slides carrying the sections embedded in L R White resin were treated as follows:-

1) Placed in 0,5% periodic acid for fifteen minutes;

2) Washed in running water for ten minutes;

3) Placed in Shiff's reagent (Appendix II) for fifteen minutes;

4) Rinsed in distilled water;

5) Destained for two minutes in 2% potassium bisulphite solution;

6) Washed in running water for thirty minutes;

7) Placed in 1% aniline blue black in 7% acetic acid at 50 - 60°C for ten minutes;

8) Dipped briefly into 7% acetic acid to remove excess dye;

9) Air dried and mounted in DPX.

Controls.

PAS reaction control:- As a control against the occurrence of free aldehydes in the tissue, L R White Resin sections were stained omitting the periodic acid oxidation step (Southworth, 1973).

Aniline blue black control:- L R White sections were subjected to a deamination process (Jensen, 1962) which removes α-amino and α-carboxyl groups from amino acids. The control slides were treated in the following manner:-

1) The sections were placed in deamination fluid (Appendix II) at room temperature for twenty four hours;

2) Washed in tap water;

3) Placed in 1% aniline blue black in 7% acetic acid at 50 - 60°C for ten minutes;

4) Dipped briefly in 7% acetic acid to remove excess dye;

5) Air dried and mounted in DPX.

Test for Tannins.

Sudan Black B Test Reaction (Pearham and Kaustinen, 1976).

Slides carrying L R White Resin sections were stained with periodic acid-Schiffs as described above (p. 70, points 1-6). The slides were then

1) Placed on wet filter paper in a large Petri dish.

2) The sections were carefully flooded with a drop of saturated Sudan Black B in 70 % alcohol. The Sudan Black B was filtered directly from a millipore syringe.

3) Staining was for an hour.

4) The staining solution was drawn off using filter paper strips and simmultaneously replaced with 70% alcohol until all the excess Sudan Black B was removed.

5) Sections were then mounted in Karo syrup.

General Staining Techniques.

Safranin and Fast Green.

Slides carrying L R White Resin embedded material were treated as follows:-

1) Placed in Safranin (Appendix II) for a minimum of sixteen hours;

2) Rinsed twice in 95% alcohol for approximately three minutes in total;

3) Placed in Fast Green (Appendix II) for thirty to sixty seconds;

4) Passed the slides through absolute alcohol; 50% absolute alcohol: 50% xylol; xylol in one minute stages.

5) Mounted in DPX.

CHROMOSOME STUDIES.

In an attempt to determine the chromosome numbers of the gametophyte and sporophyte tissue, squashes of the tissues were stained using acetocarmine, Dyers orcein and Feulgen. The results presented in this dissertation were obtained using the Feulgen technique as described by Dr Jong (University of Abberdene, Scotland; personal communication). Since none of the results obtained using the acetocarmine and Dyers orcein staining techniques are included in this dissertation, these methods will not be described.

Feulgen Technique.

The feulgen technique is ackowledged to be a specific test for deoxyribonucleic acid (DNA) and, since DNA is one of the principal components of all chromosomes, for chromosomes. It is a specific chemical reaction which involves hydrolysis of the DNA by HCl to expose the free aldehyde groups which are then able to react with the basic fuchsin. By following the protocol described below, the chromosomes and nuclei stain a deep magenta colour while the other cell components remain unstained. The intensity of the colour is proportional to the amount of DNA present.

The young gametophyte and sporophyte tissues were treated in the almost same way. Initially the material from the two generations was pretreated with 70% (aq) α-Bromonapthalene, the gametophyte tissue for ninety minutes and the sprophyte tissue for three hours. The material was then rinsed in three changes of distilled water over a fifteen minute period. The material was fixed in Farmer's (Appendix II) for thirty minutes and then washed in three changes of distilled water, each wash being for five minutes. The fixed material was then placed in a closed container containing 5N HCl for thirty minutes. During the hydrolysis step, the material was agitated gently every five to ten minutes. To remove all traces of the acid, the material was washed for fifteen minutes in three changes of distilled water and blotted on filter paper between each wash. The material was transferred to a closed container containing Feulgen's reagent (Appendix II) for sixty minutes. The stained material was placed on a clean glass slide in a drop of 45% acetic acid. The material was gently macerated, covered with a clean number one coverslip and gently squashed between two wads of filter paper. The slide was passed over a flame once or twice to enhance the colour reaction. Once the preparation appeared to be sufficiently squashed when viewed with the microscope, the slide was sealed with nail varnish and viewed.

THE DETERMINATION OF THE ORIENTATION OF MICROTUBULES USING IMMUNOFLUORESCENCE MICROSCOPY.

Immunofluorescence microscopy enables one to visualise the overall organisation of the microtubules and this can be much more useful than the information obtained from sectioned material. The method employed in this study is a combination of the methods used by Haung *et al* (1990), Sylvester *et al* (1989) and Doonan *et al* (1985).

For this experiment the spores were plated into Repli dishes containing liquid Knops medium. One of the Repli dishes was then covered with red cellophane and placed under the same culture conditions as the others (p. 65). The red light enhances one dimensional filamentous growth of the protonemata.

The protonemata grown under white light (short filaments) and those grown under red light (long filaments) where treated as follows:-

1) They were transferred to microtubule stabilising buffer (MTSB) (Appendix II) for fifteen minutes;
2) This solution was replaced by MTSB containing 2% GTA and 1% (v\v) DMSO. The protonema were fixed for a minimum of two hours;
3) Washed three times in MTSB, the last wash being overnight;
4) Placed in EGTA buffer (Appendix II) containing 2% Cellulase for thirty minutes;
5) Rinsed in three changes of MTSB over a fifteen minute period;
6) Placed in MTSB containing 5% (v/v) DMSO and 1% (v/v) Triton X for two hours;
7) Washed three times in MTSB over a fifteen minute period;
8) Washed twice in blocking solution (Appendix II), with each wash being for ten minutes;
9) This solution was drained off and replaced with a 1:100 dilution of a monoclonal antibody raised in rats against yeast tubulin (clone Y34; Kilmartin, MRC molecular biology laboratory, Cambridge, United Kingdom) for an hour. The antibody was diluted in blocking solution.

10) After three five minute washes in blocking solution, the material was incubated in fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin diluted to 1:100 with blocking solution for one hour;

11) Rinsed three times in blocking solution;

12) Individual protonemata were separated out and mounted in antifade mountant on clean glass slides;

13) The material was examined on a Zeiss Axiophot microscope with the following fluorescent attachments:-

a) exciter filter BP490

b) dichroic mirror FT510

c) barrier filters LP520 and K560

This combination provides broad excitation in the 460 - 490nm range with the beam splitting at 510nm and the detection fluorescence emission in the 520 - 560nm range, i.e. in the range of the FITC tag.

EXAMINATION OF CELL WALL DEPOSITION.

In an attempt to show that the fern protonemata exhibit an apical growth pattern, whole mounts of filamentous prothalli were made and stained for cellulase deposition using the Calcofluor White staining technique.

The liquid culture medium in which the filamentous prothalli were growing was removed and replaced with culture medium containing 0.2% (w/v) Calcofluor White. After a ten minute incubation period, the samples were washed three times in fresh nutrient medium over a six minute period. A subsample of the treated prothalli were then viewed under a microscope with fluorescence optics. The filter combination that was used was appropriate for the detection of fluorescence emission in the 390 - 420nm range. The other filamentous prothalli were placed back in the growth chamber and examined for new cell wall deposition using fluorescent

microscopy after a period of twenty four hours (Heslop-Harrison and Heslop-Harrison, 1991; Heslop-Harrison and Reger, 1988).



CHAPTER 4

RESULTS

THE SPORE.

Surface Features.

The gametophyte generation of the fern life cycle is initiated with the production of spores in the sporangium (*Plate 1, Fig. 1*).The spores of *C. viridis* var. *glauca* are approximately 46.5 ±2.26 μm in diameter and have a distinct trilete ridge (*Fig. 6; Plate 1, Fig. 2*). The spores appear to be broadly obovate in shape when viewed dorsally (*Plate 1, Fig. 2*) and semi-circular when viewed laterally (*Plate 1, Fig. 3*).The scanning electron micrographs shown in *Plate 1* show the cristate ornamentation of the perispore clearly.

Internal Spore Structure.

Despite the lengthy fixation, dehydration and embedding protocol followed, the spores did not fix, embed or section well. This is believed to be due to the presence of a very thick exospore which prevented the fixative and other solutions from infiltrating into the cytoplasm of the spore. In most instances, this poor fixation resulted in the contents of the spores falling out of the sections as they were cut. From some of the monitor sections and ultrathin sections which did remain intact, it can be seen that the spores contain a nucleus and a relatively small vacuole (*Plate 2, Fig. 1*), but that the majority of the spore volume is occupied by large vesicles which appear to be lipid bodies (*Plate 2, Figs 2 & 3*). The size of these lipid vesicles varies with the smallest ones being located towards the periphery of the spore and the largest towards the centre of the spore.

In *C. viridis* var. *glauca* the exospore is smooth. The clear band just above the level of the plasmalemma, arrowed in *Plate 2, Figs 2 & 3*, may be an inner exospore layer although it could

be an artifact of fixation. This could not be verified due to the relatively few instances where complete sections with the contents of the spore attached to the spore wall were obtained. The number of layers comprising the exospore could therefore not be determined. The exospore is believed to be composed primarily from an acetolysis-resistant carotenoid containing polymer known as sporopollenin (Raghavan, 1989).

The perispore of *C. viridis* var. *glauca* is made up of two layers. The inner perispore layer is firmly attached to the exospore. It has a granular appearance and an irregular outer margin (*Plate 2, Figs 2 - 4*). The outer perispore often detaches during the processing of the spores for electron microscopy (*Plate 2, Figs 2 - 5*). This layer is thicker than the inner perispore layer and is made up of plates which anastomose and thereby create small interconnected cavities. The way in which these plates anastomose gives rise to the cristate ornamentation seen on the surface of the spores of *C. viridis* var. *glauca* (*Plate 2, Fig. 5*), i.e. unlike most ferns, in *C. viridis* var. *glauca*, the surface ornamentation of the spore is as a result of perispore ornamentation rather than the exospore. The exospore layer is, however, responsible for the formation of the trilete ridge (*Plate 2, Fig. 1*).

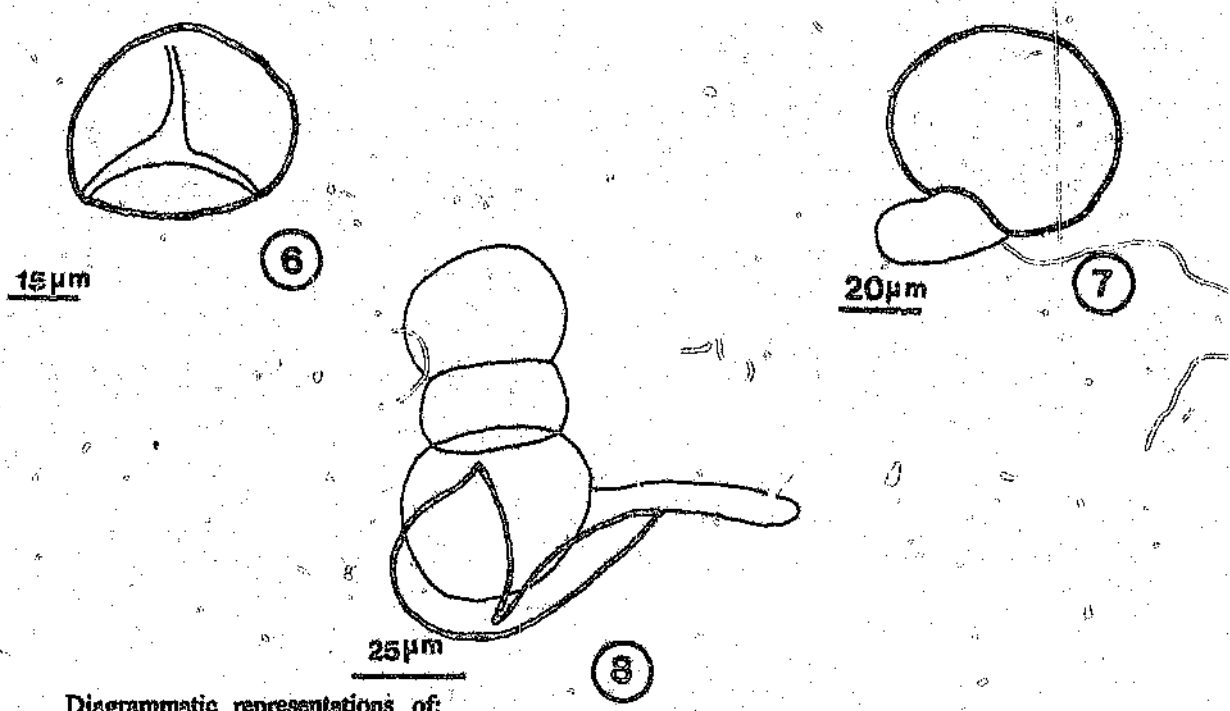
DEVELOPMENT OF THE FERN GAMETOPHYTE.

Germination.

Once imbibed, the spores of *C. viridis* var. *glauca* appear circular and have a diameter of approximately 59μm i.e. they swell to 1.3 times their original size. Nomarski interference mioroscopy of the imbibed spores shows them to be packed with vesicles, the majority of which appear to be lipid bodies (*Plate 3, Fig. 1*).

Germination of *C. viridis* var. *glauca* spores on liquid Knops medium is initiated after three to four days in culture. It is important to realise that in these experiments the spores did not all germinate together and that the rate and extent of development was not always equal. The

period given for the transition from one developmental stage to the next is therefore an average and would not necessarily be the same if the experiment were repeated.



Diagrammatic representations of:

Figure 6:- A spore of *C. viridis* var. *glauca*.

Figure 7:- The emergence of the first rhizoid from the spore.

Figure 8:- A filamentous prothallus.

The first visible sign of germination is the emergence of the rhizoid (*Fig 7; Plate 3, Fig. 2*). The rhizoid initial extends and the emerging rhizoid causes the spore wall to split along the trilete ridge (*Plate 3, Fig. 3 - 5*). Most of the cytoplasmic contents of the spore remain within the gametophyte initial (*Plate 3, Fig. 4 & 5*).

The extension of the vacuole as the rhizoid elongates affects the distribution of the cytoplasmic organelles in the rhizoid. The cytoplasm is restricted to a thin peripheral band (*Plate 4, Fig. 1 - 3*). Towards the middle of the rhizoid and at the tip region there is more cytoplasm present (*Plate 4, Figs. 2 & 4*) and the rhizoidal nucleus is found in the central cytoplasmic region (*Plate*

4, Figs. 2 - 4). The nucleus of the rhizoid is spindle-shaped to elliptical (*Plate 4, Fig. 5*). Large lipid droplets are often visible in the thickened cytoplasmic regions (*Plate 4, Fig. 6*).

The rhizoid remains unicellular and continues to elongate, often reaching great lengths in liquid culture.

One Dimensional Prothallial Growth

The prothallial initial emerges after five to six days in culture (*Plate 5, Fig. 1*) and gives rise to a uniseriate, elongated germ filament composed of chlorophyllous cells (*Fig. 8; Plate 5, Figs 2 - 4*) with one or more rhizoids at the basal end (*Plate 5, Figs 2, 3, 5 & 6*). The rhizoids emerge only from the cell of the filament closest to the spore. While the rhizoids are colourless, the protonema are bright green due to the presence of numerous chloroplasts in these cells.

All the cell divisions are by cross walls perpendicular to the longitudinal axis of the filament. The length reached by the filaments is variable but they are never less than two cells long (*Plate 5, Fig. 2*) and seldom reach lengths of more than eight cells (*Plate 5, Fig. 4*). It takes approximately eight days in culture for the filamentous stage of development to be reached.

In the apical cell of the filaments the chloroplasts were often seen to radiate in strands from a central, probably nuclear, region (*Plate 5, Figs 5 & 6*). The developmental significance of this chloroplast arrangement was not established.

The extension of the filamentous prothalli is believed to be via apical growth i.e. elongation and cell division only takes place in the apical cell of the filament. In an attempt to prove that this is the growth pattern that occurs in the prothalli of *C. viridis* var. *glauca* the prothalli were stained with Calcofluor White.

Calcofluor White is a fluorescent dye which binds to cellulase. When initially immersed in the Calcofluor White, one would expect the entire cell wall of the prothalli and the rhizoid to fluoresce (*Fig. 9*). From *Plate 6, Figs 1 & 2* we can see that this is indeed what happened.

Theoretically, as the filament elongates and continues to grow, the new cell wall which is laid down should not fluoresce since the cellulase in the new cell wall will not have been exposed to Calcofluor White (*Fig 10*). However, results clearly showing apical growth by this means were difficult to obtain. Once the prothalli had been viewed using fluorescent microscopy, no further growth of the prothalli was observed and it was therefore assumed that the ultra violet light

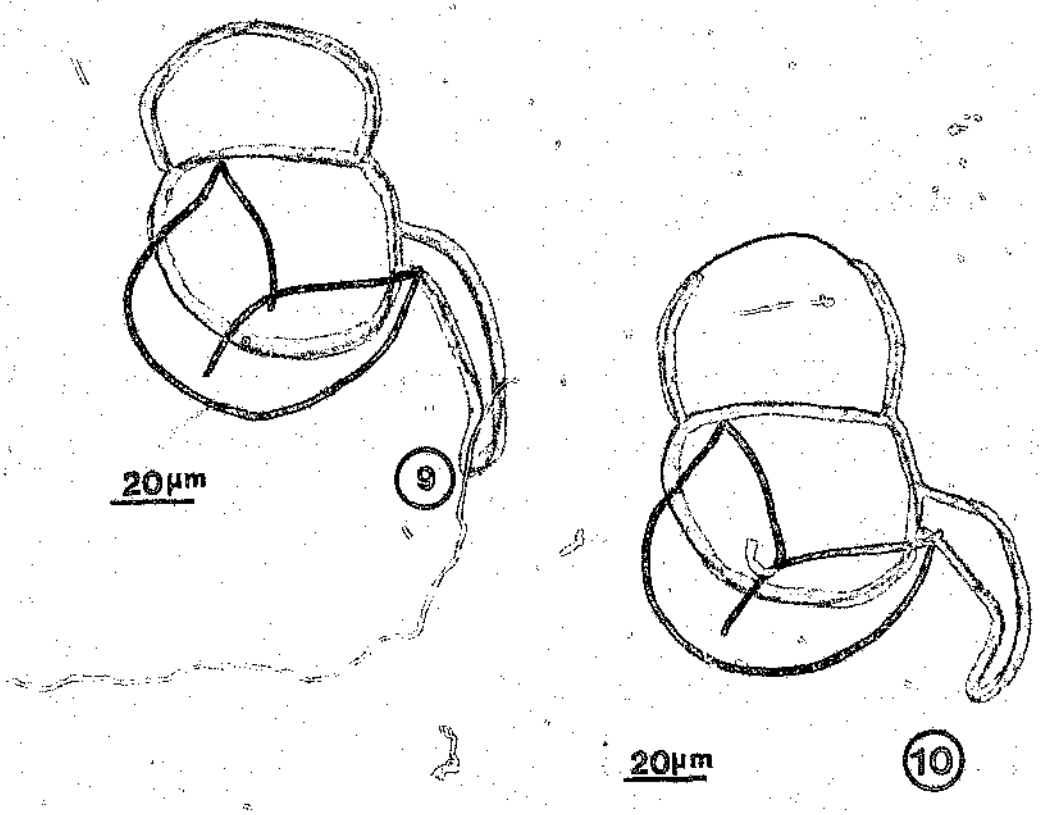


Figure 9:- When initially exposed to Calcofluor White, the entire wall of the prothallus fluoresces.

Figure 10:- The new wall laid down at the tip of the filament does not fluoresce because it has not been exposed to Calcofluor White.

inhibited elongation and cell division. The prothalli were then treated as described in the materials and methods i.e. after being incubated in Calcofluor White and washed in fresh nutrient medium, subsamples of the prothalli were viewed, photographed and then discarded. The rest of the prothalli were viewed after a twenty four hour period in an attempt to detect new cell wall deposition. The chloroplasts autofluoresced. This together with the rapid fading of the dye (*Plate 6, Figs 3 & 4*) made the detection of new cell wall deposition difficult. The Calcofluor White treated prothalli elongated very slowly and were never seen to undergo division, indicating that perhaps the dye affected the cell metabolism in some way and inhibited normal cell division and elongation. Due to these factors, no conclusive evidence that the prothalli of *C. viridis* var. *glauca* exhibit an apical growth pattern could be obtained from this study.

Two Dimensional Prothallial Development.

Six to seven days after germination (i.e. when the filaments are well developed) an abrupt change in the plane of cell division occurs. At this point longitudinal divisions are initiated and two dimensional growth of the prothallus was observed. In *C. viridis* var. *glauca* the terminal cell of the germ filament divides resulting in the formation of a wall oblique to the longitudinal axis of the filament. This is followed by a second division which is perpendicular to the first division. A transverse row of three daughter cells is formed, the middle one of which is usually wedge-shaped (*Plate 7, Fig. 1*). This is the meristematic region and all subsequent divisions are by walls parallel to the oblique walls, each being perpendicular to the one preceding it (*Plate 7, Fig. 2*). The wedge-shaped meristematic cell remains present for some time (*Plate 7, Figs 3 - 5*), but subsequent division eventually results in the formation of a group of initial cells (*Plate 7, Fig. 6*).

The daughter cells expand and by repeated transverse and longitudinal divisions form an expanded two dimensional prothallial plate which is only one cell thick (*Plate 7, Figs 3 - 7*). The

last cells to show the transition to two dimensional growth are the basal cells of the filament (*Fig. 11; Plate 7, Figs 4 & 6*). Once these cell plates have been formed rhizoid development is no longer restricted to the basal cells of the filament, but can occur from cells higher up as well (*Plate 7, Figs 2, 6 & 7*).

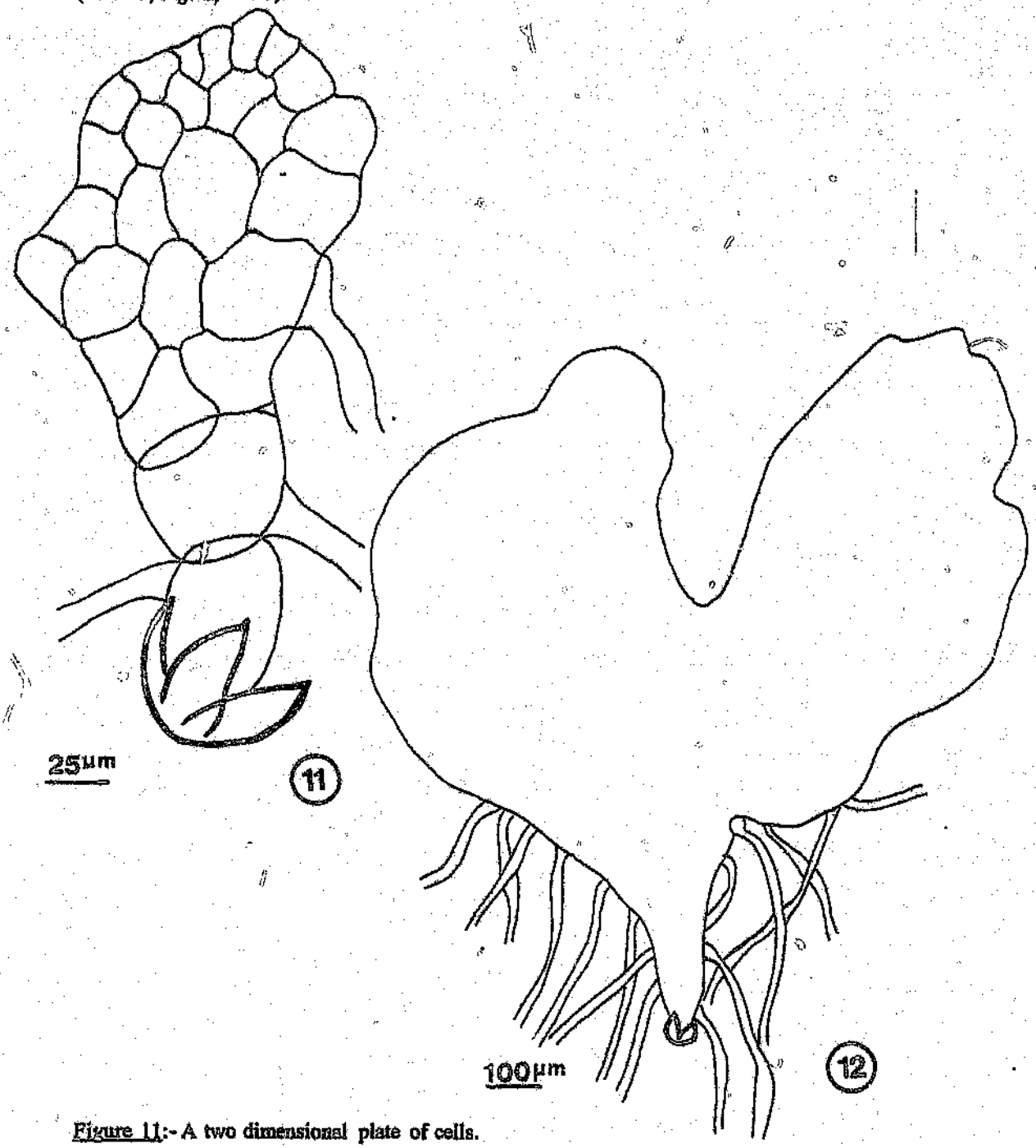


Figure 11:- A two dimensional plate of cells.

Figure 12:- A cordate prothallus.

Cordate Prothallial Development.

Eighteen to nineteen days after the onset of two dimensional growth, the prothallus shows signs of becoming notched (*Plate 8, Figs 1 - 3*). The notch develops because the cells in the region of the meristem are much smaller than those of the surrounding prothallus and are very closely packed together. Continued development in this area results in the formation of an apical notch (*Plate 8, Fig. 4*) and as a result a cordate prothallus is formed. The mature gametophytes are usually symmetrical with a median midrib and more-or-less semicircular lateral wings (*Fig. 12; Plate 8, Fig. 5*). Rhizoids occur on the dorsal surface of the prothallus and are usually restricted to the midrib area posterior to the apical notch. It takes approximately thirty days from the onset of germination for this stage of development to be reached.

A series of experiments to gain support for the theories that implicate microtubule arrangements in the transition from one dimensional to two dimensional growth were undertaken. It was anticipated that circumferentially arranged microtubules would be detected in the cytoplasm of the apical cell of the filamentous prothalli and a random arrangement of microtubules in the two dimensional prothallial plates of *C. viridis* var. *glauca*. However, demonstration of the microtubular arrangements proved elusive. The results obtained are shown in *Plate 9*.

An adapted method of Doonan *et al* (1985) using indirect immunofluorescence microscopy was adopted for this study. The control slides showed a large amount of autofluorescence caused by the presence of numerous chloroplasts within the cells of the filamentous prothalli (*Plate 9, Fig. 1*). In the experimental slides, the autofluorescence obscured most or all of the microtubule fluorescence (*Plate 9, Fig. 2*). The elongated filaments, produced by growing the prothalli under red light, showed less autofluorescence in the cells away from the apical cell since in these cells the chloroplasts were smaller and more spread out. However, as *Plate 9, Fig. 3* shows, these larger cells appeared to collapse during preparation and no distinct microtubule arrangements

were discernible. The apical cells of these long filaments also contained a large amount of chlorophyll and therefore the microtubule fluorescence was obscured by the large amount of autofluorescence (*Plate 9, Fig. 4*). Possible ways of dealing with the problems encountered with using the indirect immunofluorescence technique for localising microtubules will be dealt with in the Discussion (p. 97). Since the use of this technique on the one dimensional filamentous prothalli proved unsuccessful, the technique was not attempted on the two dimensional prothallial plates.

Ultrastructure of the Gametophyte Cells.

Throughout the development of the gametophyte the ultrastructure of the cells remains more-or-less constant.

Typically a large vacuole occupies half or more of the cell volume (*Plate 10, Figs 1 & 2*). The vacuole is surrounded by a tonoplast.

The nucleus is usually situated towards one side of the cell and is often seen in a resting phase with clumped heterochromatin in the nucleoplasm giving the nucleus a fine granular appearance (*Plate 10, Figs 3 & 4*). A single spherical nucleolus is usually present (*Plate 10, Fig. 4*) and the dark regions seen within the nucleus is heterochromatin. The nucleus is surrounded by a double nuclear membrane (*Plate 10, Fig. 5*).

Mitochondria and dictyosomes are located uniformly throughout the cytoplasm of the gametophyte cell (*Plate 11, Figs 1 - 3*). The matrix surrounding these organelles consists predominantly of tubules of endoplasmic reticulum and numerous small vesicles (*Plate 11, Figs 3 & 4*). The structure of the mitochondria resembles that of higher plants, i.e. they are bounded by a double unit membrane, the inner one being extensively folded to form cristae. The cristae

are embedded in a dense stroma. The many small granules in the stroma are mainly ribosomes while the few larger granules are electron dense granules (*Plate 11, Fig. 2*).

Myelin-like structures (*Plate 11, Figs 5 & 6*) are commonly observed within the cytoplasm of the gametophyte cells. Although the exact function of these structures is not known it is assumed that in fast-growing cells such as these with a high membrane turnover, they would fulfil a generally accepted lysosomal function.

Chloroplasts are abundant in the cells of the gametophyte. Surface views of the gametophyte cells at the light microscope level show the chloroplasts forming an almost complete covering around the cells (*Plate 12, Fig. 1*) and sections through the filamentous gametophytes confirm that these chloroplasts are primarily peripherally situated (*Plate 12, Fig. 2*). Electron microscopy showed the chloroplast profiles to be approximately 4μm in length and 2.8μm in breadth (*Plate 12, Figs 3 & 4*). Each chloroplast is surrounded by a double unit membrane - the chloroplast envelope (*Plate 12, Fig. 5*). The photosynthetic lamellae are of varied lengths and occur either singly as stromal lamellae or aggregated into grana (*Plate 12, Fig. 6*). The lamellae tend to lie in the direction of the longitudinal axis of the chloroplast (*Plate 13, Fig. 1*). Osmiophilic droplets are commonly observed within the stroma of the chloroplast (*Plate 12, Figs 3, 5, & 6; Plate 13, Figs 1 & 4*).

Starch grains within the chloroplasts are numerous and variable in size. Their shape is usually ellipsoidal or spindle-like (*Plate 13, Figs 2 & 4*). Mitochondria are often seen in close association with the chloroplast (*Plate 13, Fig. 5*).

Microtubules were occasionally seen, occurring in triplets, between the chloroplasts and the plasmalemma (*Plate 13, Fig. 3*).

DEVELOPMENT OF THE APOGAMOUS SPOROPHYTE.

In *C. viridis* var. *glauca* no antheridia or archegonia are formed and the sporophyte is produced apogamously.

After roughly forty five to forty eight days in culture changes begin to occur in the region of the apical notch (*Plate 14, Fig. 1*) and a meristem arises (*Plate 14, Figs 2 & 3*). From the light microscope observations made, the origin of the meristem could not be traced to one particular cell of the gametophyte but rather to a group of cells in that region. These initial cells are similar to the adjacent cells of the prothallus except they are greatly reduced in size (*Plate 14, Fig. 4*). Mitosis takes place in rapid succession in this meristem resulting in plates of cells being laid down on the surface of the prothallus (*Plate 14, Figs 5 & 6*). With continued division a thick cushion-like region is formed at the base of the apical notch (*Plate 15, Figs 1 & 2*).

This cushion or mound of tissue, referred to as the apogamous bud, from which the sporophyte develops takes approximately five days to develop. Arising from the apogamous bud, are numerous multicellular hairs (*Plate 15, Figs 3 & 4*). As the size of the apogamous bud increased so did the size and number of hairs surrounding it (*Plate 15, Figs. 5 & 6*). The function of the hairs is not known but may be one of protection. The apical cell of the hairs often appeared orange.

The mature apogamous bud appears as a dark bottle-green [illegible] cells at the base of the apical notch (*Plate 16, Fig. 1*). Within five to eight days from the onset of apogamous bud development the root and shoot apices were seen to differentiate almost simultaneously (*Plate 16, Fig. 2*). However, the root of the sporophyte only completed its development at a much later stage. The first leaf develops and grows out from the apogamous bud (*Plate 16, Figs 3 & 4*). Often elongated structures developed from the bud region - these elongated structures grew

along the surface of the nutrient medium and appeared to contain vascular tissue although were never seen to form sporophytes (*Plate 16, Fig. 5*).

At this stage the multicellular hairs were still evident at the junction of the leaf and apogamous bud (*Plate 17, Fig. 1*), on the petiole of the leaf (*Plate 17, Figs 2 & 3*) and on the leaf surface itself (*Plate 17, Figs 3 & 4*).

Vascular tissue which is a distinguishing character of the sporophyte generation of the ferns, is well developed by this stage, most notably the tracheids (*Plate 17, Figs 5 & 6*).

More leaves were produced and only once two or three leaves were well established (*Plate 18, Figs 1 & 2*) did the root develop fully (*Plate 18, Figs 3 & 4*). At this point the sporophyte was able to support itself and the gametophyte became necrotic and died. The first leaf grew straight out of the apogamous bud with only the lamina of the leaf showing a slightly circinate pattern of development. All the subsequent leaves produced showed the typical, very tight, circinate pattern of growth (*Plate 18, Fig. 4*).

Once two to three leaves of the sporophyte were well established, the ferns were transferred from the liquid culture medium to beakers containing a 50:50 mixture of potting soil and vermiculite where they were allowed to lead an independent existence. It took approximately two hundred days of culturing before the ferns were large enough to be transplanted into the beakers of soil.

Ultrastructure of the Apogamous Sporophyte.

Although the apogamous bud is embedded in gametophyte tissue, the cells making up the apogamous region, and later the sporophyte, can be clearly distinguished from those of the vegetative gametophyte (*Plate 19, Figs 1 & 2*). The first most noticeable difference between the

cells of these two regions is the difference in size. The cells of the gametophyte are much larger (*Plate 19, Fig. 4*) than those of the apogamous bud and developing sporophyte. The cells giving rise to the apogamous sporophyte have a large, usually centrally situated nucleus (*Plate 19, Fig. 3*) and the cells appear to contain more chloroplasts per unit volume than the vegetative gametophyte cells (*Plate 19, Figs 3 & 4*). There is a distinct meristematic region towards the tip of the apogamous outgrowth (*Plate 19, Fig. 6*). The cells in this region have the major part of the cell volume occupied by the nucleus.

What is of particular interest in the cells from which the sporophyte arose is the presence of a dark, osmiophilic layer at the level of the tonoplast (*Plate 20, Figs 1, 2 & 4*). This layer was absent in the meristematic cells of the apogamous outgrowth (*Plate 20, Fig. 3*) as well as from the cells of the multicellular hairs which surround the developing apogamous bud (*Plate 20, Fig. 5*). A very thin layer is found in the gametophyte cells immediately adjacent to the apogamous bud (*Plate 20, Fig. 6*) while gametophyte cells further away from the bud did not have the tonoplastic layer present at all.

The darkly staining deposits are seen in the vacuoles of the cells of the apogamous bud at about the time the multicellular hairs appear around the apogamous bud (i.e. about fifty to fifty three days after germination). This material was gradually deposited as "droplets" on the tonoplast (*Plate 21, Figs 1 - 4*) until, in the very mature apogamous bud and the developing sporophyte a complete layer forms on the tonoplast (*Plate 21, Figs 5 & 6; Plate 22, Figs 1 & 3*). The deposition of this material in these cells results in a distinct boundary between the gametophyte and sporophyte tissues (*Plate 22, Figs 2, 4 & 5*). The plastids of cells possessing this layer are poorly developed with no distinct thylakoids present (*Plate 22, Fig. 3*).

In an attempt to determine what this layer is composed of various histochemical procedures were carried out on sectioned material. The general stain toluidine blue stains the particles

within the vacuole a dark blue and served to prove that these layer-forming particles occur in the vacuole of the cell (*Plate 23, Fig. 1*). However, it did not reveal the chemical composition of the layer. The combined test for protein and polysaccharides using the aniline blue black and periodic acid-Schiffs reaction was negative. While the starch grains in the chloroplasts stained pink (appearing red/purple with Nomarski interference optics) with the periodic acid-Schiffs stain (*Plate 23, Fig. 2*) and the proteins in the nucleus stained blue with the aniline blue black, the particles within the vacuoles of the cells of the apogamous bud did not take up either of the stains (*Plate 23, Figs 3 - 5*). This therefore indicates that this material is neither a protein nor a polysaccharide.

The control slides for PAS (*Plate 23, Fig. 1*) showed only the nuclei staining blue due to the aniline blue black. No organelle staining was observed in the aniline blue black controls (*Plate 23, Figs 2 & 3*).

When stained with safranin and fast green, these layer-forming particles stain a deep purple although this colour came out a pinkish-brown/ red in the photographs taken using bright field optics and dark purple-blue with Nomarski interference optics (*Plate 24, Figs 1 - 3*). This result therefore indicates that the material has an acidic component to it.

The particles in the vacuole of the apogamous bud cells stain light yellow with Sudan Black B. With Nomarski interference optics these particles appear pale pink (*Plate 24, Figs 4 & 5*). This positive reaction indicates that the layer formed in these cells is a tannin. The protocol followed for staining the sections with Sudan Black B caused the sections to lift off the slides, fold and crinkle, making it very difficult to take completely focused photographs of the sections since they were not lying flat on the slides.

To correct the distortion of the colour of the stained tissue due to the use of a tungsten filament in the microscope, a daylight filter and correct colour film exposure time should be used. Due to the lack of filter combinations on the microscope used the colour distortion factor could not be corrected.

Chromosome Studies in Gametophyte and Sporophyte Tissue.

Following the method described on page 72 chromosome spreads for the gametophyte tissue were obtained in very young two dimensional prothallial plates (*Plate 25, Fig. 1*) *Plate 25 Figs 2 - 4* show a through focus of a gametophyte cell with a good spread of chromosomes. From these photographs, and others similar to them, it was determined that the gametophyte cells of *C. viridis* var. *glauca* have sixty chromosomes.

Despite numerous attempts no chromosome spreads were obtained for the sporophyte generation. Squashes of young apogamous bud material as well as material taken from the first sporophyte leaf did not yeild any chromosome spreads. However, a comparison of *Plate 25, Fig. 5*, which shows the nucleus of a gametophyte cell in interphase, and *Plate 25, Fig. 6*, which is the nucleus of a sporophyte leaf cell, the dimensions of the nuclei and the amount of nuclear material within the nuclei are very similar and may be indicative of equal chromosome numbers.

CHAPTER 5

DISCUSSION

THE SPORE.

Surface Features.

In the Pteridophytes spore wall structure and surface features are generally remarkably stable (Tryon, 1985) and, along with other characteristics of ferns, are often useful in depicting generic or subgeneric groups. Fine details of the spore have been investigated with the scanning electron microscope to provide new characters for use in the classification of the cheilanthoids (Tryon and Tryon, 1973).

One of the first observations made regarding the spores was that there are marked differences in the sizes of cheilanthoid spores. In the largest there is a correlation with a reduction in the number of spores per sporangium that reflects the apogamous condition; for example the very large spores of the apogamous fern *Pellaea atropurpurea* (L) Link may be up to 92µm in diameter. The spores of *Chellanthes feei* Moore, *Notholaena aurea* (Poiv.) Desv. and of *Cheiloplecton rigidum* (Sw) Fee. are equally large and these species have a reduced spore number; this information suggests that they are apogamous (Tryon and Tryon, 1973). The size of the spores of *Chellanthes viridis* var. *glauca* are, however, much smaller than those mentioned here, being only approximately 46,5µm, yet this study has shown that *C. viridis* var. *glauca* is an apogamous fern. It would therefore appear that the size of the fern spore is not a reliable characteristic in determining whether or not a fern is apogamous.

From Tryon and Tryon's (1973) study of cheilanthoid spores it is evident that the cristate form of sporoderm (≡ perispore) predominates. The cristate ornamentation, occurring in *C. viridis* var. *glauca*, typically consists of more or less disconnected, irregular ridges as seen in the

scaning electron micrographs in *Plate 1*. This form of perispore ornamentation occurs in widely diverse species of the cheilanthoids and is also characteristic of several species groups e.g. it occurs in several species of *Dryopteris* J. Sm. and is the uniform type among the light-petiolated species of *Pellaea* Link.

The other types of perispore patterning observed by Tryon and Tryon (1973) in the cheilanthoids are:-

a) Rugulose:- Rugulose sporoderm, composed of low, more or less compact rugae occurs in several groups of cheilanthoids e.g. *Cheilanthes lendigera* (Cav.) Sw., *Pellaea calomelanos* (Sw.) Link and *P. ternifolia* (Cav.) Link. The rugulose type seems to have a close relationship to the cristate and species such as *Mildella intramarginalis* (Link) Trev. have spores intermediate between these two forms. The prevalence of cristate and rugulose sporoderm, and intermediate forms, suggest that they are basic types among the cheilanthoid ferns.

b) Verrucate:- Verrucate spores with large, low rounded tubercles are distinctive and characterize a group among the American species of *Notholaena* R. Br. This unique form of sporoderm suggests that this group of species has probably originated independently of other cheilanthoids.

c) Reticulate:- Reticulate spores in several species have diverse kinds of networks e.g. the spores of *Mildella smithii* (C. Chr.) Hall and Lell. have delicate muri fused with coarser rugae while the spores of *Aspidotis meifolia* (D.C. Eaton) Pic. Ser. are uniformly reticulate but with irregularly projecting muri. In *Cheilanthes argentea* (Gmel.) Kze. the muri are strongly anastomosing.

d) Echinate:- The echinate form is one of the most distinctive types of sporoderm e.g. the structure in *Pellaea paradoxa* (R. Br) Hook. consists of coarse attenuated protruberances.

e) Granulose:- Granulose sporoderm occurs in spores of *Notholaena aurea* (Poir.) Desv. and

Cheiloplecton rigidum (Sw.) Fee. In both species the spores are extremely large, and the granular surface seems to overlay coarser regulose or echinate material which suggests that they may represent specialised forms.

The frequency of rugulose and cristate forms of perispore through a broad spectrum of the cheilanthoids suggests that these represent some basic spore types of species derived from a common ancestral group. Other forms of the sporoderm, such as the echinate, verrucate, reticulate or granulate, may represent specializations of the rugulose or cristate form, or types representing other evolutionary lines.

The surface structure of the sporoderm is uniform within most species-groups (Tryon and Tryon, 1973) and is therefore a useful characteristic for classification purposes.

Internal Spore Structure.

The preparation of sporogenous material for both light and electron microscope studies is known to be difficult (Beisvåg, 1970) mainly due to the thick, hard spore walls which are relatively impervious to both fixatives and resins. As noted in the results (p. 76), a clear indication of inadequate infiltration of both fixatives and embedding material in the spores of *C. viridis* var. *glauca* was during the preparation of a cutting face on the ultramicrotome when the contents of the spores dropped out (*Plate 2, Figs 4 & 5*). Parkinson (1980) experienced similar problems when fixing the young sporangia of *Psilotum nudum* (L.) Beauv. In an attempt to overcome this problem the spores were processed using a prolonged period of fixation and infiltration in Spurr's resin. Although infiltration was successful in that penetration of spores by the embedding medium was almost complete, the fixation of the spore cytoplasm was poor. The poor fixation was probably due to the slow penetration of the fixative through the spore wall (*Plate 2, Figs 1 - 3*).

The structure of the spore wall (*Plate 2*) in *C. viridis* var. *glauca* is similar to that of *C. odora* Sw. as described by Lugardon (1974). Both these species of ferns have a smooth, blechnoid exospore with few noteworthy features and a thick, complex perispore. Lugardon (1974) found that in *C. odora* the perispore was composed of two layers - the inner layer was 0.4μm thick, more or less granular in texture and has an irregular outer margin. This layer is firmly attached to the exospore in *C. viridis* var. *glauca* (*Plate 2, Figs 2 - 4*).

The outer perispore layer is thicker than the inner layer (in *C. odora* it measures between 0.5μm - 1.5μm) and is composed of more or less discontinuous thin plates, which anastomose, thereby creating many small interconnected cavities joining to each other and to the external environment. These cavities in the outer perispore layer of the spores of *C. viridis* var. *glauca* are most clearly evident in *Plate 2, Fig. 4*. The cristate ornamentation seen on the surface of the spores of *C. viridis* var. *glauca* is due to the anastomosing plates of the outer perispore layer.

The internal organisation of organelles in the spores of *C. viridis* var. *glauca* is similar to that found by Gantt and Arnott (1965) in their study of germination in *Matteuccia struthiopteris*. Details of their findings are given in the literature review (p. 9).

DEVELOPMENT OF THE FERN GAMETOPHYTE.

Primary Rhizoid Development.

Of the three patterns of spore germination described by Nayar and Kaur (1971) *C. viridis* var. *glauca* follows the category known as polar germination most closely. To recap from the literature review, polar germination is when the first cell division in the germinating spore is by wall formation parallel to the equatorial plane of the spore, elongation of the primary rhizoid and young thallus is parallel to the polar axis of the spore (Nayar and Kaur, 1971). From *Fig. 2* on page 8 one can see that in this pattern of germination the rhizoid grows out more or less

perpendicular to the filamentous prothallus and parallel to the polar axis of the spore. This feature is clearly illustrated in *Plate 5. Figs 2, 5 & 6.*

A detailed study of the ultrastructure of the rhizoid of *C. viridis* var. *glauca* was not undertaken. From the gross morphological observations made, the internal organisation of the organelles of the rhizoid appear to be similar to those described in other fern species such as *Dryopteris bornei* Kuntze (Dyer and Cran, 1976). The vacuole is visually the most dominant feature of the rhizoid (*Plate 4*) with the cytoplasm being located primarily at the tip of the rhizoid and in a region approximately half way down the rhizoid. The nucleus is spindle-shaped and occurs within the central cytoplasmic region.

Development of a Prothallus.

Gametophyte development in *C. viridis* var. *glauca* clearly follows the *Adiantum*-type of development described by Nayar and Kaur (1971). The development of the uniseriate, elongated germ filament composed of barrel-shaped chlorophyllous cells and bearing rhizoids only from the basal cell (shown in *Plate 5*) is more or less standard for a number of ferns (Miller, 1980; Nayar and Kaur, 1971; Davis, 1969; Whittier, 1965). The development of the gametophyte from this stage until the formation of the heart-shaped gametophyte differs within the Pteridophyte - a detailed description of the various types of prothallial development which occur, as described by Nayar and Kaur (1971), are given in the literature review (p. 24 - 27).

It is generally accepted that the protonemata of ferns exhibit tip/apical growth (Ito, 1969; Ootaki, 1968). The attempt to show this in *C. viridis* var. *glauca* using Calcofluor white as an indicator of where new cell walls were being deposited was unsuccessful. However, a distinctive feature of apical growth is that since elongation does not occur in the daughter cells basal to the division plane, the cell length of these cells is fixed by the position where cell division occurs in the parent apical cell (Ootaki, 1968). No actual measurements were necessary as it was

obvious that only the apical cell of the filamentous protonemata of *C. viridis* var. *glauca* appeared to elongate and "grow"while the cells posterior to this cell remained the same length, indicating that the filamentous protonemata exhibit apical growth.

The results illustrated in *Plates 6* and *7* clearly indicate that further development of the gametophytes of *C. viridis* var. *glauca* follow the *Adiantum*-type of development i.e. the apical cell divides in such a way that a wedge-shaped meristematic cell is formed. Continued division of the wedge-shaped meristematic cell, and later the group of meristematic cells which develop from this single cell, results in the formation of a two dimensional prothallial plate. As in *Pteris vittata* L., once two dimensional growth has been initiated, the development of rhizoids is not restricted to the basal cell but occurs from other cells of the thallus as well (Cohen and Crotty, 1990). Further development in the region of the meristem, causes the prothallus to become notched and in the mature gametophyte the fully developed apical notch results in the formation of a symmetrical cordate prothallus with semicircular lateral wings.

The Use of Immunofluorescence Microscopy to Determine the Role of Microtubules in the Transition from One Dimensional to Two Dimensional Growth.

The transition from one dimensional to two dimensional growth is thought to involve the microtubular cytoskeleton of the cells. In the apical cell of the filamentous prothalli (which exhibits one dimensional growth) the microtubules of the cortical cytoplasm can be divided into two groups with one set being aligned parallel to the longitudinal axis of the cell and the other set circumferentially around the cell at the region of the dome. In prothalli exhibiting two dimensional growth patterns, the cortical microtubules were found to be randomly orientated (Wada *et al*, 1990; Murata *et al*, 1987).

The attempt to prove that such microtubular arrays occur in the one dimensional and two dimensional prothalli of *C. viridis* var. *glauca* using indirect immunofluorescence microscopy

proved unsuccessful due to a number of factors. From this study it can be concluded that before indirect immunofluorescence microscopy can provide evidence of the microtubule arrays found in the apical cells of the filamentous prothalli of *C. viridis* var. *glauca* the autofluorescence of the chloroplasts in these cells will have to be cut down or completely eliminated by e.g. using a different antibody conjugate such as rhodamine conjugates since it has been found that much autofluorescence occurs at the optimum excitation wavelength of FITC; not fixing the material in glutaraldehyde or formal acetic alcohol since these fixatives produce non specific tissue fluorescence; or using additional filter combinations simultaneously with the fluorescent filters (Dr Wolfe-Coote, Tygerberg Hospital, Cape Town; personal communication).

Once the autofluorescence problem has been solved, the method may need to be adapted to optimise infiltration of the antibody and fluorescent conjugate into the prothallial cells and to eliminate background fluorescence before good microtubule arrangements can be observed. Dr Doonan (John-Innes Institute, London; personal communication) indicated that achieving good, repeatable results using the indirect immunofluorescence method is time consuming and expensive. It was therefore felt that, since the issue of the role of the microtubules in the transition from one dimensional to two dimensional growth was undertaken out of interest rather than as a central issue in this dissertation, time should not be spent in perfecting the technique. This aspect of the dissertation may form the basis of another study.

The ultrastructure of the gametophyte cells of *C. viridis* var. *glauca* show no marked differences from the ultrastructure of other ferns that have been studied (Cran, 1979; Wada and O'Brien, 1975; Gantt and Arnott, 1965).

THE DEVELOPMENT OF THE APOGAMOUS SPOROPHYTE.

In the majority of ferns once the mature heart-shaped gametophyte has formed, antheridia and archegonia develop towards the base of the gametophyte between the rhizoids and behind the

apical notch respectively. Fusion of the gametes results in the formation of an embryo and the sporophyte develops from the embryo.

However, in *C. viridis* var. *glauca* there was no evidence of the development of antheridia and/or archegonia but rather the sporophyte developed from the gametophyte apogamously. No addition of nutrients such as glucose, sucrose or fructose, or growth hormones such as gibberellic acid or ethylene to the nutrient media were required to induce the development of the apogamous bud and water was not a limiting factor, therefore, it can be concluded that under the culture conditions used, *C. viridis* var. *glauca* is an obligate apogamous fern.

In the sexually reproducing ferns there are a number of steps which always occur prior to the development of the sporophyte. One of the initial triggers in sexual reproduction is the induction of antheridium formation by the endogenously produced antheridiogens (Näf, 1969). At more or less the same time archegonia develop on the shaded surface of the prothallus behind the apical notch. On contact with water sperm are released and the swiming sperm are attracted to the archegonia by mucilagenous protoplasmic material released during the opening of the archegonia (Whittier and Peterson, 1980; DeMaggio, 1977). The ordered development of the sporophyte follows fertilization of the egg by a sperm cell.

In the apogamous ferns there are no obvious or characterised triggers which give an indication as to when apogamous bud development will be initiated. Unlike the ferns which reproduce sexually, no set developmental scheme of apogamous bud formation or sporophyte development has been recorded for apogamous ferns.

While it is generally agreed that the apogamous sporophyte develops from a thickened region just posterior to the base of the apical notch on the shaded side of the prothallus (Raghavan,

1989; Smith, 1938; Verdoorn, 1938), there is some dispute regarding the exact origin of the apogamous bud.

Duncan (1941) in his study of apogamy in *Doodia caudata* traced the origin of the meristem which gives rise to the apogamous bud to a three-sided apical cell which develops posterior to the apical notch. In Yamanouchi's (1908) description of the origin of the apogamous sporophyte in *Nephrodium* (*Dryopteris*) *molle* the apogamous bud was believed to originate from a single cell on the ventral side of the prothallium a short distance back from the apical notch. This cell could be identified due to its larger size, larger nucleus and denser contents.

Duncan (1943) (quoted in Steil, 1951) studied a number of apogamous fern species and in all instances traced the origin of the apogamous sporophyte to a number of cells of the gametophyte rather than to one single cell. Smith (1938) also noted that, while in some instances the apogamously produced sporophyte derives from a single cell of the gametophyte (either a vegetative cell, a component of of the archegonium or the egg of the archegonium (parthenogenesis)), in other cases the apogamously produced sporophytes are first evident as a compact mass of cells on the ventral side of a gametophyte.

Whittier (1962; 1965; 1970) (summarised in Rashid, 1976) described in detail the origin and development of apogamous sporophytes on *in vitro* cultured gametophytes of *Pteridium* Scop. and *Cheilanthes* Sw.

In Whittier's (1970) study of *Cheilanthes castanea* Sw. the first indication of the origin of apogamous form was traced to a meristematic centre in the thickened part of the prothallus. He found the meristem to originate from a single cell or from a group of two to three cells. The initial cell(s) were simila. to prothallial cells in the sinus of the gametophyte (*Fig 13.1 & 13.2*). The first division of the cell(s) was periclinal (*Fig 13.3*) and the daughter cells produced

were similar to the prothallial cells except for the presence of small chloroplasts (*Fig 13.4 & 13.5*).

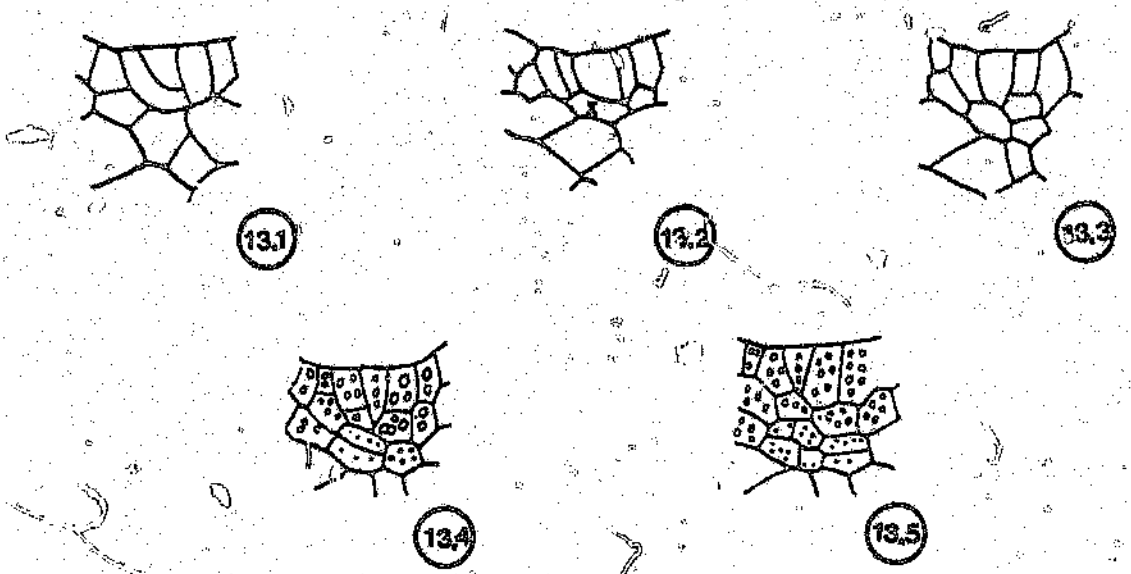


Figure 13.1 - 13.5:-Diagrams of portions of the gametophyte of *Cheilanthes catanea* near the apical region showing the origin of the apogamous sporophyte from a single cell (marked with a cross). The sporophyte cells can be distinguished from the other cells of the gametophyte by their smaller chloroplasts (adapted from Whittier, 1970; Rashid, 1976).

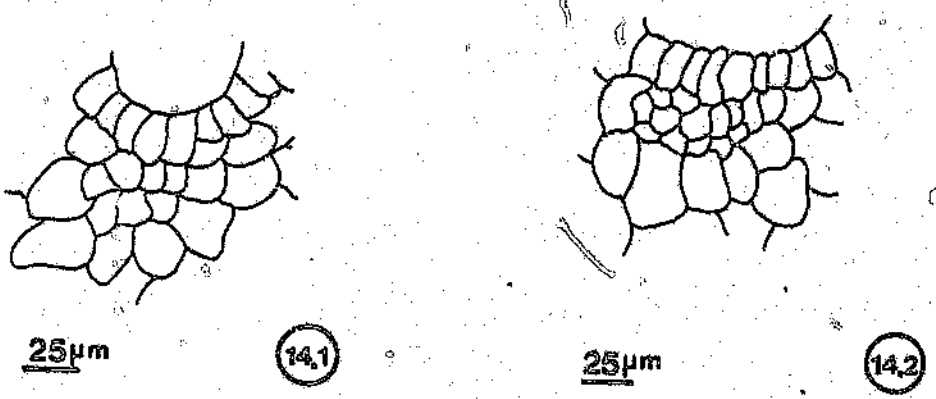


Figure 14.1 - 14.2:-Diagrammatic enlargements of Plate 14, Figs 2 & 3 showing the region below the apical notch beginning apogamous bud formation in *C. viridis* var. *glauca*.

Diagrammatic representations of *Plate 14, Figs 2, 3 & 4 (Fig 14.1, 14.2 & 14.3)* greatly resemble the diagrams presented by Whittier (1970) showing the origins of the apogamous sporophyte in *C. castanea*. From the photographs and diagrams made of *C. viridis* var. *glauca* the origin of the apogamous sporophyte can be traced to two to three cells which differ from the surrounding gametophyte tissue only in size.

Steil (1939) noted that the young embryo in apogamous ferns, frequently before the first leaf has made its appearance, is surrounded by multicellular hairs and also, frequently by scales. In agreement with this observation the glandular, multicellular hairs are clearly evident surrounding the developing apogamous embryos of *C. viridis* var. *glauca* (*Plate 15, Figs 4, 5 & 6*)and are still evident on the petioles and leaf surface of the primary leaf (*Plate 17, Figs 1 - 4*).

DIFFERENCES BETWEEN SEXUALLY REPRODUCING FERNS AND APOGAMOUS FERNS.

The literature indicates that there are a number of significant differences between ferns which produce sporophytes sexually and those showing apogamy:-

According to the literature one of the major differences between apogamous ferns and those reproducing sexually is that the apogamous embryo is intimately connected with the prothallium in such a way that one cannot decide where one begins and the other ends (Steil, 1951; Yamanochi, 1908).

Those periods of the life cycle of homosporous ferns when cells are changing their manner of growth are marked by the cells concerned being surrounded by conspicuous barriers e.g. in sporogenesis the wall enveloping the spore mother cell is impregnated with callose, a polysaccharide which appears to be a widespread sealing agent in the plant kingdom. This wall does not break down until the newly formed spore has aquired at least some exine and its

gametophytic nature is probably largely established. In spermatogenesis the walls around the differentiating spermatocytes are similarly callosed (Bell, 1979).

In oogenesis the wall of the archegonial chamber loses its plasmodesmata and also becomes transiently callosed. During the phase of nucleocytoplasmic interaction, possibly the occasion on which the newly activated sporophytic genes impress themselves upon the female cytoplasm, the egg becomes surrounded by an acetolysis-resistant membrane, which is likely to be impermeable to all but the simplest metabolites (Bell, 1979; Bell, 1961). This membrane, which occurs at the level of the plasmamembrane, persists during fertilization, continues to surround the zygote, and its attenuated remains are clearly seen in the common wall between zygote and young embryo.

The occurrence of these barriers of reduced or negligible permeability is understandable in the light of the hypothesis of cyclic alternation. The preparation of a cell, within the tissue of its parent, for a different phase of growth may indeed be impossible if it continues to suffer the ingress of informational molecules such as polynucleotide and ribonucleotide acids. From the view point of the life cycle, the barriers which are significant are those around the spore mother cell, in relation to the change from sporophyte to gametophyte, and maturing eggs, in relation to the converse. Therefore at each transition the beginning of the alternative phase of growth is a well protected event in the tissue of its parent. When the new phase of growth finally emerges from its envelope it is evidently fully stabilized (Bell, 1979).

According to the literature there is no such barrier in apogamous ferns and one cannot initially distinguish the sporophytic generation from the gametophytic generation (Steil, 1951; Yamanouhi, 1908). The study of *C. viridis* var. *glauca* does not indicate this continuity. *Plate 19* shows that the sporophyte tissue and the gametophyte tissues are easily distinguishable from one another and a closer examination of the cells forming the interface between the sporophyte and

gametophyte showed the presence of darkly staining particles in the vacuoles of these cells (*Plates 20 - 22*). The presence of this layer at the level of the tonoplast appears to form a barrier between the two generations - possibly equipollent to the layer which surrounds the maturing egg in the ferns which reproduce sexually. One very important difference between these two layers is that in the sexually reproducing ferns the layer occurs at the level of the plasmamembrane while in *C. viridis* var. *glauca* the layer forms at the level of the tonoplast. Because no autoradiographic studies were performed on sections through the apogamous bud of *C. viridis* var. *glauca* to indicate whether this layer does form a barrier to nutrient movement between the two generations as in the sexually produced embryos, no conclusive statement regarding its function in providing a protective development site for the new sporophyte can be made. However, the absence of plasmodesmatal connections between these cells lends support to this possible role in sporophyte development.

Histochemical studies performed on cells showing the presence of this layer indicated that it consists of the secondary plant metabolite tannin (a complex poly phenol) (*Plates 22* and *23* and p. 88).

Plant phenolics used to be regarded as inert end products of metabolism but radioactive tracer techniques show that they are capable of considerable interconversion. Since they have been found in almost all higher plants and in many of the lower groups, it seems unlikely that they have no function, even though they are not involved in the fundamental metabolic processes. It has been proposed that some phenolics may have no other function than to be intermediates in the biosynthesis of more complex plant products (Smith, 1976).

However, a widely supported view, and a probable one for explaining the presence of this tannin material in the vacuole of the cells surrounding the developing sporophyte in *C. viridis* var. *glauca*, is that plant phenolics play an important role in deterring would-be predators. Bate -

Smith (quoted in Smith, 1976) commented on the unpleasant astringency of developing pears. The taste is due to the presence of tannins in unripe fruits. When the seeds are fully developed, it is selectively advantageous that the fruit be edible and attractive. Presumably the high tannin concentration in young fruits are a "device"which discourages premature seed dispersal (Smith, 1976).

The action of tannins extends to viruses. Allard (1965) (quoted in Smith, 1976) showed inhibition of tobacco mosaic virus by tannic acid.

Anti-microbial and anti-herbivore activity have been demonstrated from ecological and laboratory studies for many classes of secondary compounds found in ferns (Banerjee and Sen, 1980 (quoted in Cooper-Driver, 1983)). For example, simple phenolic compounds were found to be active against both fungi and bacteria and also as actual feeding deterrents to non-adapted insects (Jones and Firn, 1979 (quoted in Cooper-Driver, 1983)). More complex phenolic polymers - the condensed tannins - were found to operate to reduce herbivory and played a significant role in the resistance of mature fronds to herbivory (Cooper-Driver, 1983).

The function of the tannin layer in the cells forming the interface between the two generations in the life cycle of *C. viridis* var. *glauca* may simply be a mechanism to prevent the herbivory of the young developing sporophyte and/or it may play a role in preventing movement of excess nutrients and information between the two generations since movements of molecules would only be able to occur via the cell wall or cytoplasm, with tonoplastic movement being inhibited/reduced due to the presence of this layer. Tracer experiments would need to be performed to determine this function.

The presence of the layer at the level of the tonoplast as well as the distinct differences between the cells of the sporophyte generation and the gametophyte generation lead to the conclusion

that in *C. viridis* var. *glauca* the apogamous embryo is not as intimately connected with the prothallium as the literature would have us believe.

Another important difference between embryos produced apogamously and those produced sexually is that no foot or equivalent structure forms at the onset of apogamous sporophyte development (Steil, 1951; Yamanoch., 1908).

It is generally accepted that in liverworts, mosses, hornworts and pteridophytes the sporophytic generation interfaces with the gametophytic generation via a foot or haustorium (Petterson and Whittier, 1991). This foot acts as an absorbing organ since the embryonic sporophytes are heterotrophic, depending on the gametophyte for carbohydrates and other nutrients (Peterson and Whittier, 1991; Gifford and Foster, 1989; Verdoorn, 1938). The ultrastructural features of the young sporophyte foot region and the adjacent gametophyte cells suggests that they are involved in short distance transport. The gametophyte cells bordering or intermingling with the haustorial cells develop extensive wall ingrowths, typical of transfer cells in higher plants (Chauhan and Schraudolf, 1985). These transfer cells have few mitochondria, irregularly-shaped plastids with little starch, a large central vacuole and the cytoplasm is restricted to a narrow layer below the cell walls. Since no plasmodesmatal connections exist between cells of the haustorial foot and the gametophyte, the transfer of water and nutrients takes place soley through the apoplast (Peterson and Whittier, 1991; Chauhan and Schraudolf, 1985).

In agreement with the literature concerning apogamous ferns, no foot or haustorium was seen to develop in *C. viridis* var. *glauca*. The cells at the interface of the gametophyte and sporophyte generations (*Plate 22*) do not show extensive wall ingrowths which are typical of transfer cells and, although the plastids were irregularly shaped with few thylakoids, they contained large compound starch grains. The large vacuoles in these cells restricted the cytoplasm to the

periphery of the cell and as described above the most significant feature in these cells was the tannin layer which occurred at the level of the tonoplast.

Another characteristic given to differentiate apogamous embryos from sexual ones is that the vascular tissue of the sporophyte is in direct connection with vessels which lie in the prothallium (Steil, 1951; Yamanouchi, 1908).

Plate 17 clearly shows that in *C. viridis* var. *glauca* the development of vascular tissue is restricted to well within the cells of the sporophyte as defined by the tannin border formed between the two generations. No vascular tissue was seen to occur within the prothallial tissue.

The order of appearance of organs from the sexual embryo and the apogamous embryo differs (Steil, 1951; Yamanouchi, 1908).

The first organ to be clearly distinguished from embryos produced via sexual reproduction is the foot. While the foot is in the process of forming the leaf initials appear in a position lateral and distal to the foot. The stem and root initials form one to two days after the differentiation of the leaf initials. Approximately thirty days after fertilization the root breaks through the calyptra and about two days later the leaf straightens out over the prostrate prothallus (Sheffield and Bell, 1987; DeMaggio, 1977).

In *C. viridis* var. *glauca* root and shoot apices differentiated almost simultaneously from the apogamous bud (*Plate 16, Fig. 2*). The primary leaf appeared first, followed by the stem initial and other leaves. The roots only developed at a later stage once two to three leaves were relatively well established. This order of appearance agrees with the observations made for other apogamous ferns (White 1979; Lawton, 1936).

The leaves of the apogamous and sexually produced sporophytes are similar with long, thin petioles and blades that only constitute a small fraction of the leaf length. The leaves are however believed to differ in the number of lobes they have with the blade of the first leaf from the apogamous sporophyte being three-lobed rather than the two-lobed condition which is typical of sexually produced sporophytes (Whittier, 1976; Duncan, 1941). *Plate 17, Fig. 3* and *Plate 18* clearly indicate that in *C. viridis* var. *glauca* the first leaf produced from the apogamous bud is two-lobed or else is entire, but in no instances are three lobes observed. This characteristic of the first leaf cannot therefore be used to distinguish apogamously produced sporophytes from those produced sexually.

GENETICS OF THE GAMETOPHYTE AND APOGAMOUS BUD.

Apogamy results in the maintenance of the sporophytic chromosome number in the gametophytic generation (Sheffield and Attree, 1983). From squashes of young gametophyte tissue a chromosome count of sixty was obtained. The numerous attempts made to obtain a chromosome count for the sporophyte generation i.e. squashes of young apogamous bud material and squashes of cells from the first leaf of the apogamous sporophyte, were unsuccessful. This was possibly due to the pretreatment time being too short (although in the final attempt, the sporophyte tissue was pretreated for three hours and still no chromosome spreads were obtained) and/or by this stage in development the cell wall was too thick for the solutions to penetrate sufficiently. Most chromosome counts of the sporophyte generation are obtained from root squashes. In this study, however, the roots of the young sporophytes were few, very small and difficult to work with. The number of times the squashes could be repeated was therefore limited and the attempts made to get chromosome counts from these roots were unsuccessful.

Since no actual chromosome count was therefore obtained for the sporophyte it cannot be said with one hundred per cent certainty that the gametophyte and sporophyte of *C. viridis* var.

glauca have the same chromosome number. However, this can be deduced from a number of factors which are evident in *Plate 25, Figs 5 & 6* and from the literature. *Plate 25, Figs 5 & 6* show the nuclei of a gametophyte and a sporophyte cell respectively. Measurements and observations made from these photographs and from numerous other sections which showed the nuclei of the respective generations it appeared that the size (32,5 ± 4,55 μm for the gametophyte and 40,3 ± 5,8 μm for the sporophyte) and density of the nuclei from the two generations are very similar therefore suggesting that the amount of nuclear material found within the nuclei are similar.

Perhaps a more relevant or reliable indication that the chromosome count of sixty obtained from the gametophyte cells is the diploid chromosome number of the sporophyte is information obtained from the literature. Walker (1973) indicates that a number of families within the Pteridophyta are cytologically uniform e.g. *Marattiacece serisu lato*, in which all the genera have been examined are based on a chromosome number of forty, or Osmundaceae in which n = 22 appears to be the invariable rule. Genera such as *Pteris* L., *Adiantum* L. and *Cheilanthes* Sw. possess chromosome numbers based on twenty-nine and or thirty. This group is referred to as the "adiantoid group". Generic records for plants belonging to the adiantoid group such as those for *Adiantopsis radiata* (L.) Fee with 2n = 60 or for *Hemionteris wernerii* Ros. with n = 29, serve to emphasize this consistency.

It would therefore appear that a haploid chromosome count of approximately thirty and a diploid count of approximately sixty would be considered the norm for the adiantoid group. Chromosome counts of other species of *Cheilanthes* also substantiate this e.g. *Cheilanthes alabamensis* (Buckl.) Kunze has a gametophyte count of thirty, *C. chipinquensis* Knobloch has a sporophyte count of fifty eight, *C. farinosa* Blandford and *C. madernsis* Lowe have gametophyte n = 30 and sporophyte n = 60 while in *C. marantae* (Cav.) ssp. *subcardata* (Cav.)

Beni. and Poelt n = 29 for the gametophyte and n = 58-60 for the sporophyte (Moore, 1967-1971).

It can, therefore, be concluded with some certainty that in the obligately apogamous fern *C. viridis* var. *glauca*, the gametophyte and the sporophyte have the diploid chromosome number of sixty.

ADVANTAGES OF APOGAMY TO *C. viridis* var. *glauca*.

One of the major advantages that the apogamous mode of reproduction offers to ferns is that water is no longer essential for fertilization to occur (White, 1979; Whittier, 1965). Considering that *C. viridis* var. *glauca* grows primarily in rocky areas such as on ledges in crevices of cliffs and at the base of rocks where water is relatively scarce, this feature of apogamy would definitely be of advantage to this fern.

Another advantage of apogamy mentioned by Anthony (1983) which applies to *C. viridis* var. *glauca* is that the elimination of sexual reproduction by apogamous plants enables them to retain all the accumulated genes which favour survival in that particular habitat i.e. to the relatively xeric conditions where this fern grows.

Being apogamous therefore enables *C. viridis* var. *glauca* to inhabit drier regions where competition from other ferns, and plants in general, for water, nutrients and space is limited.

CHAPTER 6

CONCLUSION

The primary aim of this study has been achieved. This dissertation describes in detail the development of the gametophyte of *Cheilanthes viridis* var. *glauca* from the time of spore germination to the formation of an independent sporophyte. It can be concluded from this study that, under the culture conditions used, *C. viridis* var. *glauca* is an obligately apogamous fern. The other aims (stated in full on page 2) of this dissertation have been achieved in that:-

1) The apogamous bud was found to develop from two to three meristematic cells located at the base of the apical notch;
2) A tannin layer was located at the level of the tonoplast in cells which separates the developing sporophyte from the surrounding gametophyte tissue - such a layer has not been reported previously. This layer differs from the lipophilic layer found in sexually reproducing ferns in that it does not occur at the level of the plasma membrane and may have a different function;
3) The apogamous sporophytes produced by *C. viridis* var. *glauca* differed from the sporophytes produced via sexual reproduction in other ferns in that no foot was seen to develop and the leaf was the first organ of the sporophyte to develop with root formation occurring much later in the developmental sequence.

CHAPTER 7

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PLATE 25:- Chromosome spreads and nuclei of gametophyte and sporophyte tissues 176

PLATE I

Scanning Electron micrographs of the spores of *C. viridis* var. *glauca*.

Figure 1:- Spores in a sporangium.

Figure 2:- A dorsal view of a spore showing the trilete ridge (arrowheads).

Figure 3:- A lateral view of a spore showing the cristate ornamentation of the perispore.

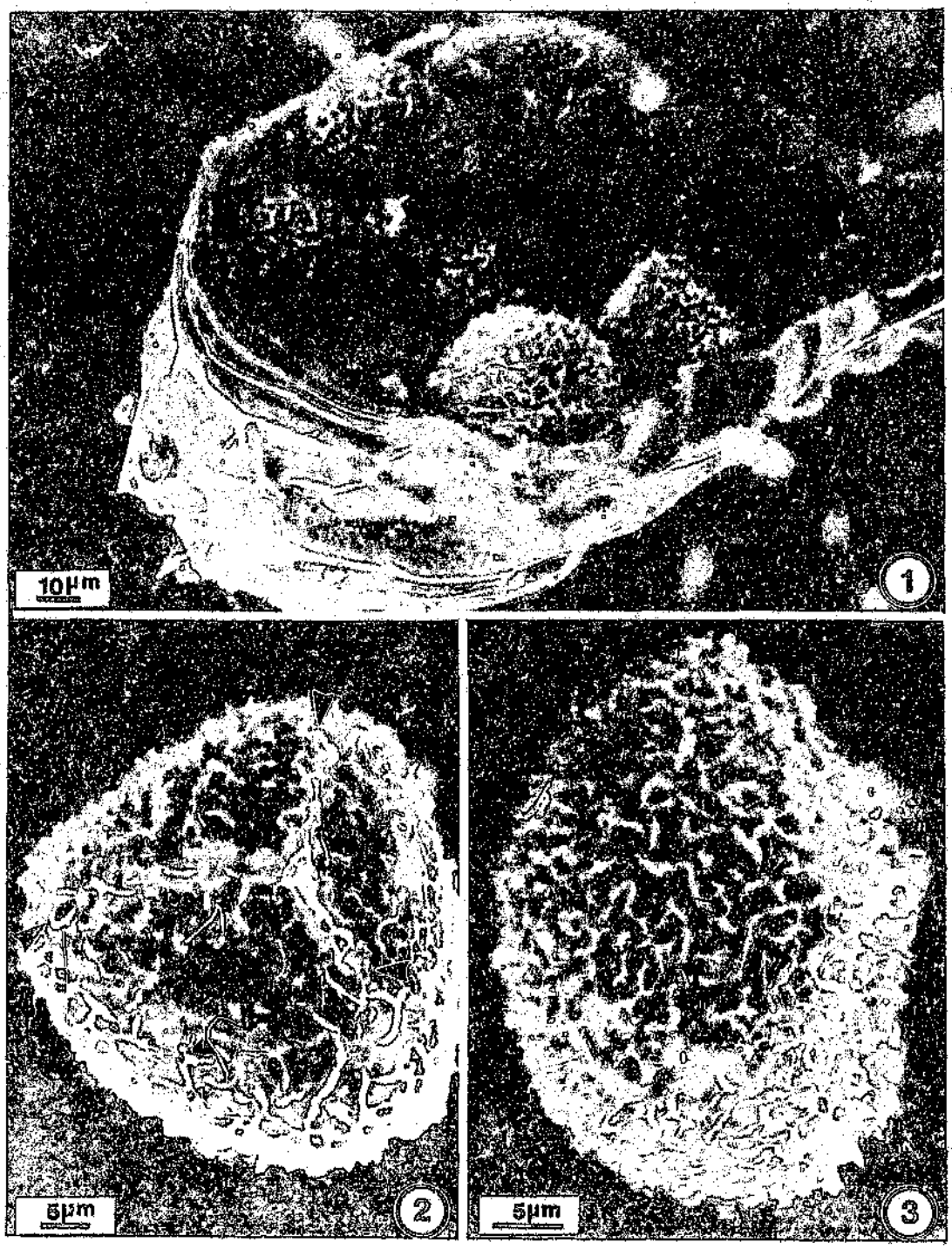

10µm
1
5µm
2
5µm
3

PLATE 2

Details of the internal structure and wall of the spores of *C. viridis* var. *glauca*.

Figure 1:- A semi-thin section through a spore showing the position of the nucleus (n), vacuole (v) and lipid bodies (l). The surface ornamentation of the spore is due to the patterning of the perispore (arrowed) while the exospore gives rise to the trilete ridge (arrowheads).

Figure 2:- The perispore consists of two layers, the outer layer (p) being less firmly attached than the inner, granular perispore layer (arrowhead). The exospore (e) is smooth and the clear layer next to the plasmalemma of the spore (arrowed) may be a second exospore layer. Lipid bodies (l) are outlined within the spore.

Figure 3:- The two perispore layers (p & arrowhead) are seen lying next to the smooth exospore (e). Small lipid vesicles are located towards the periphery of the spore (arrowed) and larger lipid bodies (l) towards the centre of the spore.

Figure 4:- The cristate ornamentation of the spore is due to the cavities formed (arrow) by the anastomosing plates which make up the outer perispore layer (p). This outer perispore layer has pulled away from the more firmly attached inner perispore layer (arrowhead). The contents of the spore (*) in this section have fallen out.

Figure 5:- Details of the plates of the outer perispore layer.

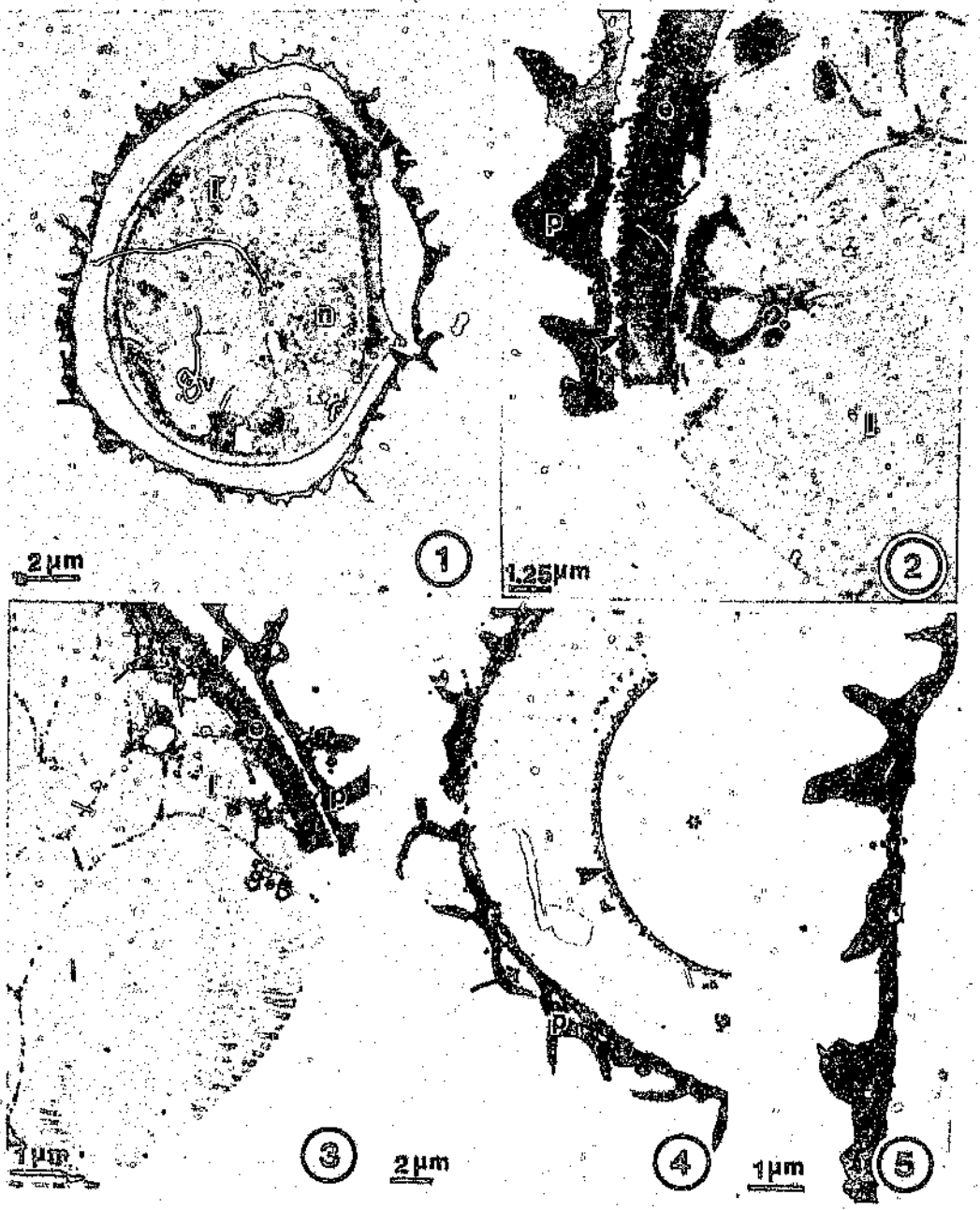

2 µm
1
1.25µm
2
1µm
3
2µm
4
1µm
5

PLATE3

Early stages in the germination of *C. viridis* var. *glauca*.

Figure 1:- Imbibed spore packed with vesicles, the majority of which appear to be lipid bodies.

Figure 2:- After three to four days in culture the rhizoid (r) emerges from the spore.

Figure 3:- Emergence of the rhizoid causes the spore wall to split along the trilete ridge (arrowheads).

Figure 4:- Lateral view showing a squashed spore. The spore coat splits along the trilete ridge.

Figure 5:- The major part of the cytoplasmic contents of the spore remains within the prothallial initial (pr).

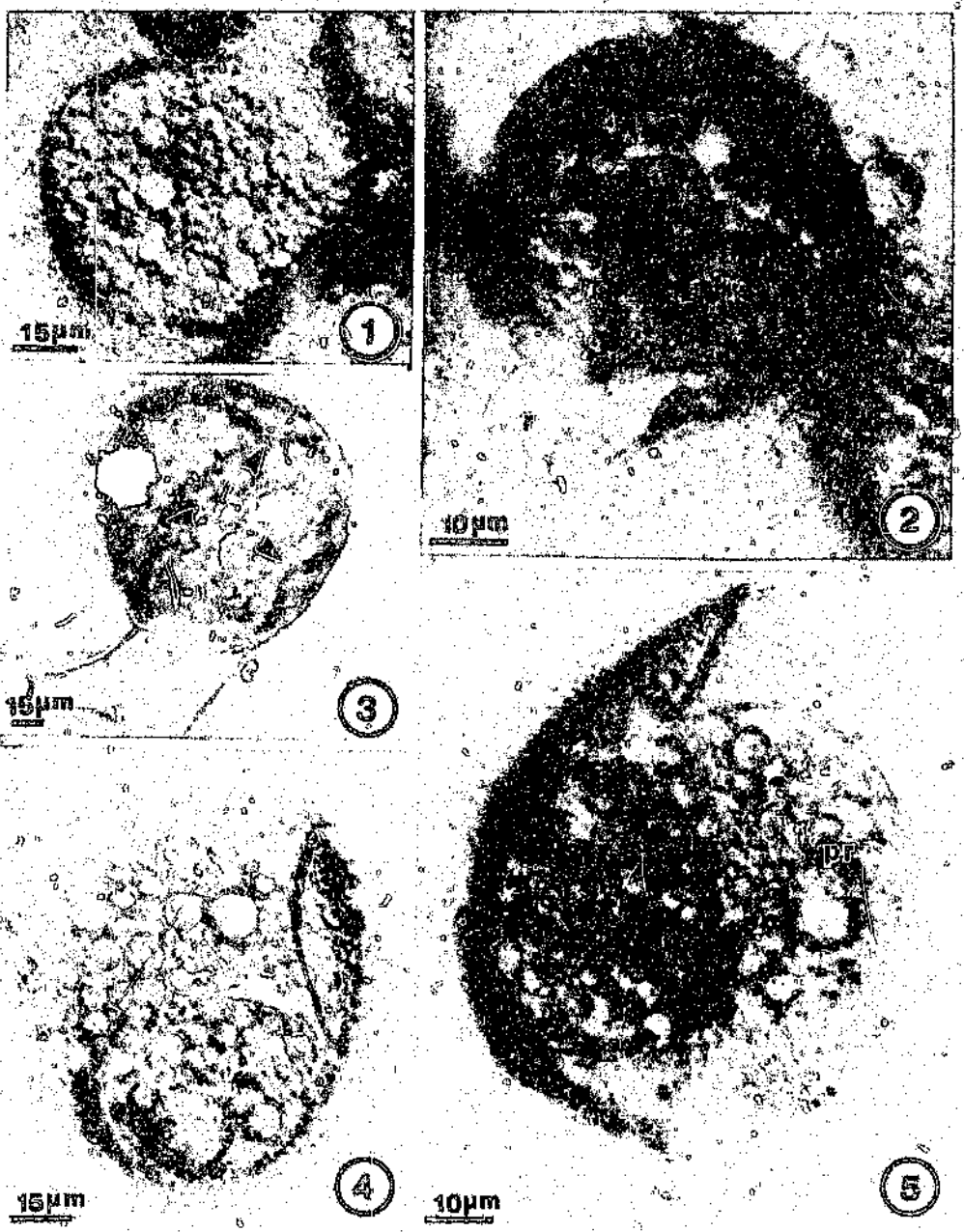

15µm
1
10µm
2
15µm
3
15µm
4
10µm
5
DF

PLATE 4

The cytoplasmic organisation of the primary rhizoid.

Figure 1:- The presence of a large vacuole (v) causes the cytoplasm to be restricted to the periphery of the rhizoid (arrowhead).

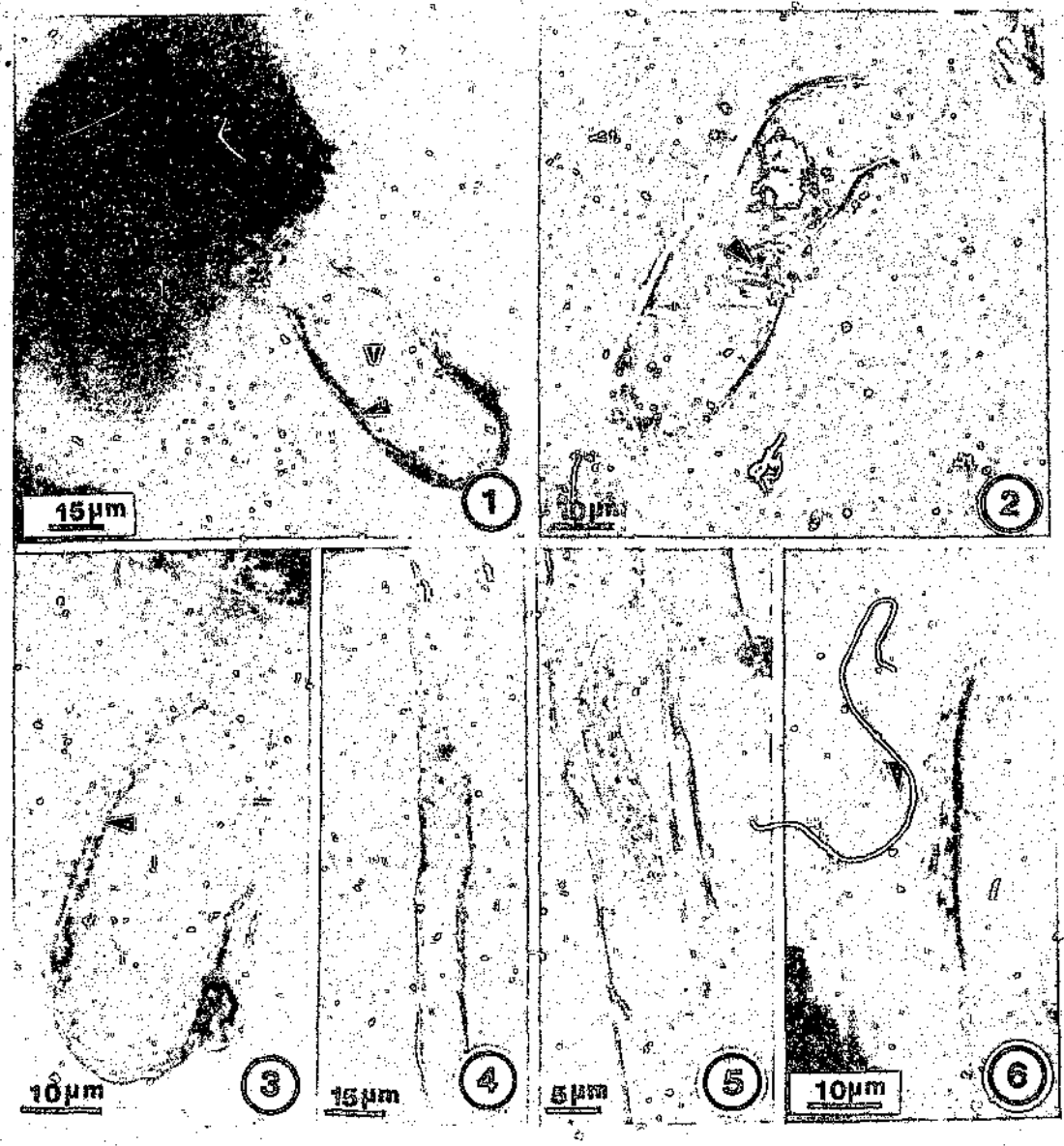
Figure 2:- In the central region and at the tip of the rhizoid (arrowheads) there is a thickening of the cytoplasm.

Figure 3:- Peripherally situated cytoplasm of the rhizoid (arrowhead).

Figure 4:- The nucleus is present in the central mass of cytoplasm (*).

Figure 5:- The spindle-shaped nucleus of the rhizoid.

Figure 6:- Lipid droplets (arrowhead) are often seen within the dense cytoplasmic regions.


15µm
1
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2
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3
15µm
4
5µm
5
10µm
6

PLATE 5

The development of a filamentous prothallus.

Figure 1:- The prothallial initial (pr) which emerges after five to six days in culture and gives rise to the prothallus.

Figure 2:- A three celled prothallus with one basal rhizoidal cell.

Figure 3:- Cell walls (arrowheads) perpendicular to the longitudinal axis of the filament indicate perpendicular cell divisions.

Figure 4:- Eight celled filament.

Figure 5:- Strands of chloroplasts (arrowhead) radiating from a central nuclear region (n) of the apical cell. The rhizoid (r) grows out perpendicular to the filamentous prothallus.

Figure 6:- The nuclear region (n) is evident in the apical cell with the chloroplasts radiating from this region (arrowhead). Lipid vesicles are evident throughout the prothallus. The primary rhizoid (r) emerges at ninety degrees to the filamentous prothallus.


1
15µm


2
15µm


3
20µm


4
20µ


n
5
15µm


n
6
15µm

PLATE 6

Apical growth in the filaments of *C. viridis* var. *glauca*.

Figure 1:- Fluorescence of the cell wall after binding of calcofluor white to cellulase (arrowheads) and autofluorescence of chloroplasts (red).

Figure 2:- The entire prothallial wall (arrowheads) fluorescing when initially exposed to Calcofluor White.

Figure 3:- Autofluorescence of the chloroplasts masks the effect of the Calcofluor White fluorescence.

Figure 4:- As the dye fades rapidly only autofluorescence of the chloroplasts is seen in this micrograph.



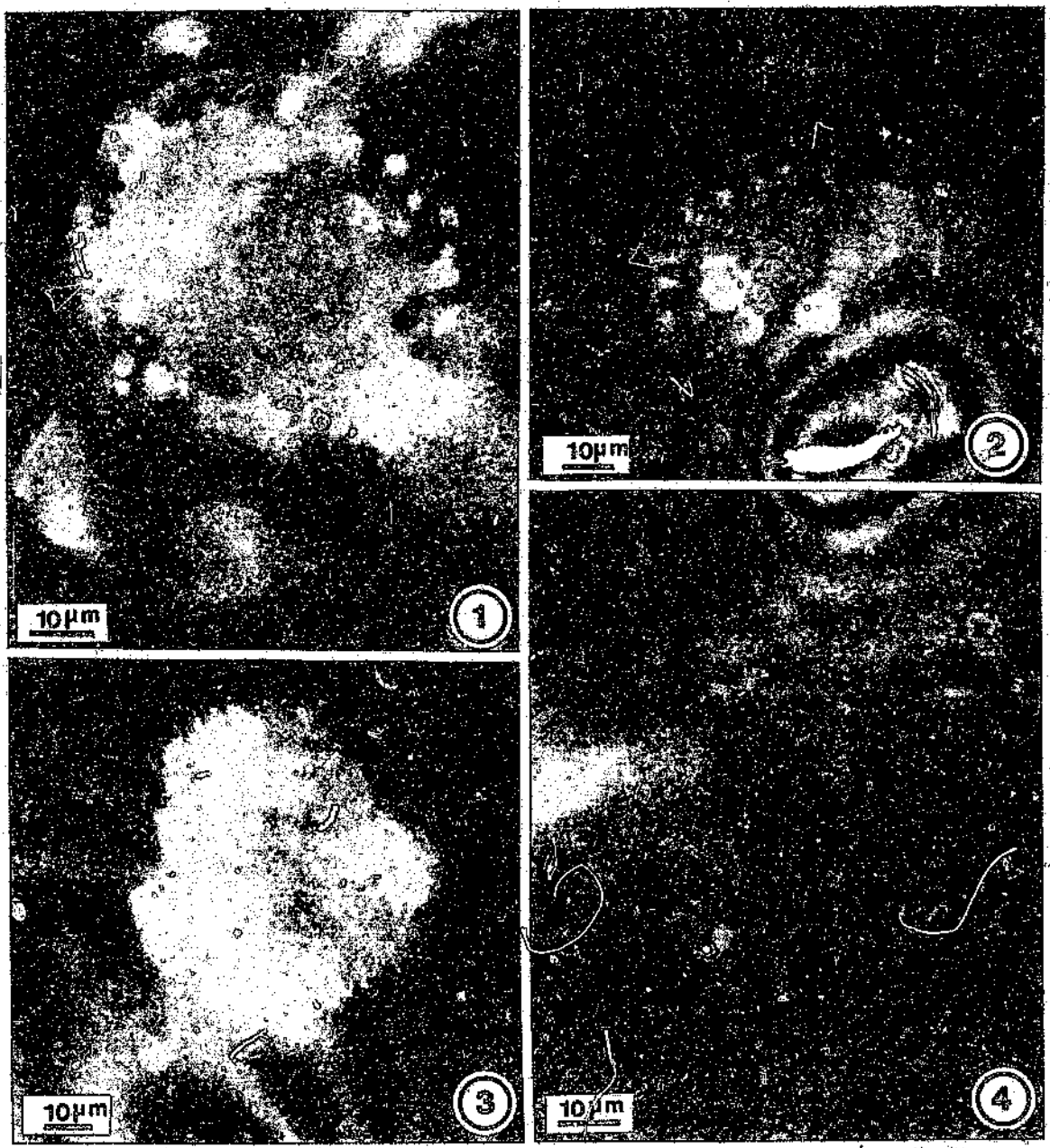

10μm
1
10μm
2
10μm
3
10μm
4

PLATE 7

The initiation of two dimensional growth.

Figure 1:- The apical region of the filament showing oblique divisions which form a wedge-shaped meristematic cell (w).

Figure 2:- The arrowhead indicates a wall formed perpendicular to the division preceding it. Rhizoids (r) are seen to arise from cells of the filament other than the most basal ones.

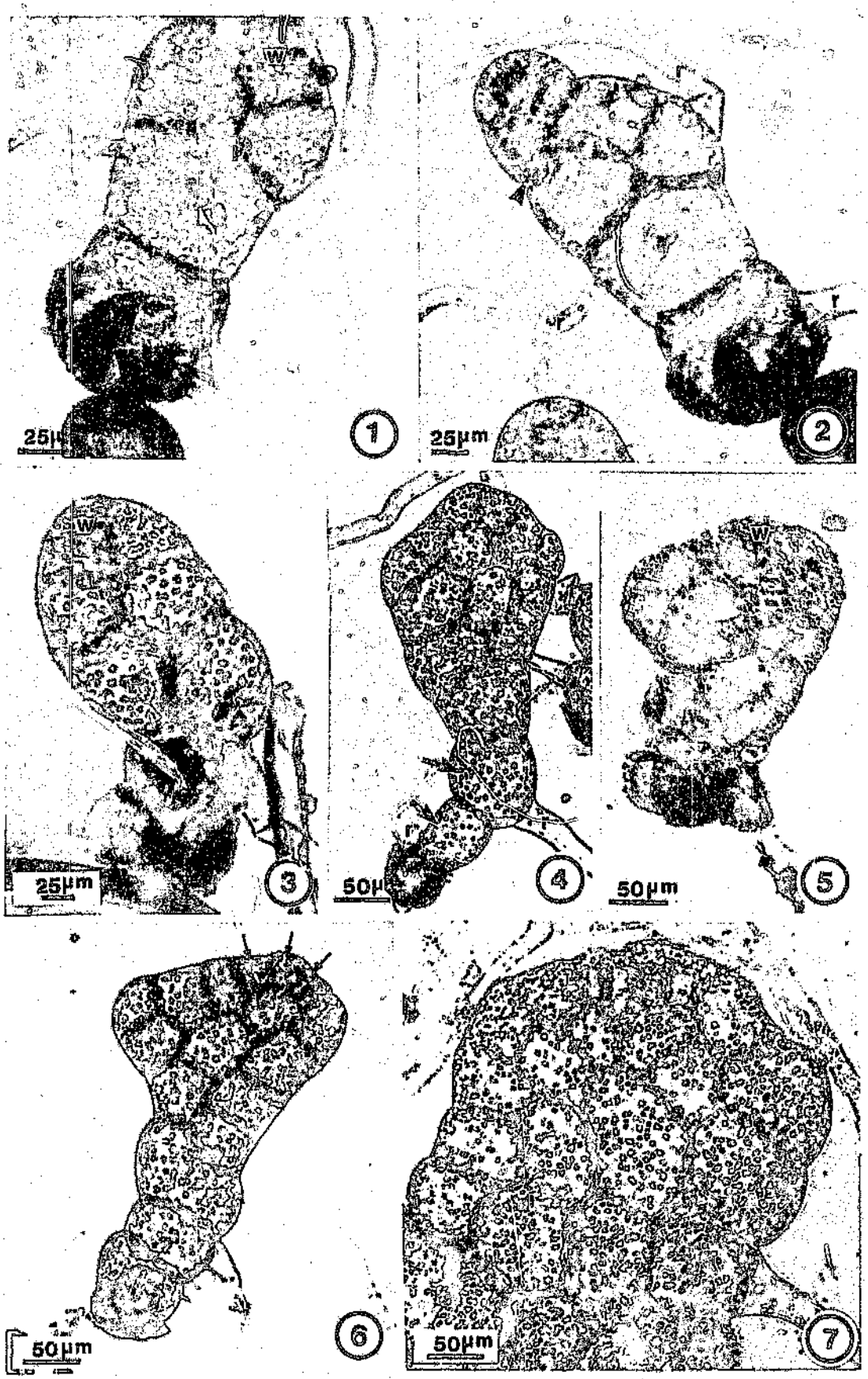
Figure 3:- Two dimensional growth continues down the filament. The wedge-shaped meristematic cell (w) is still evident at this stage of two dimensional growth.

Figure 4:- Basal cells of the filament (arrowheads) are the last to show the transition to two dimensional growth. Rhizoid (r) formation as in Fig. 2 is no longer restricted to the most basal cells of the filament.

Figure 5:- A two dimensional prothallus, taken using Nomarski interference optics, showing the wedge-shaped meristematic cell (w).

Figure 6:- A group of initial cells (arrowed) now constitutes the meristem. Basal cells of the filament still do not show two dimensional growth.

Figure 7:- A one cell thick, two dimensional prothallial plate.


25µm
1
25µm
2
25µm
3
50µm
4
50µm
5
50µm
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7

PLATES

Final stages in the development of a cordate prothallus.

Figure 1:- A notched region (arrowhead) which develops in the region of the meristem.

Figure 2:- A detailed view of the early stages of development of the notched region of the prothallus.

Figure 3:- A distinct notched region (arrowhead) is evident after the gametophytes have been in culture for approximately thirty days.

Figure 4:- The apical notch which forms due to continued development in this meristematic region.

Figure 5:- A mature gametophyte with a median midrib and semi-circular lateral wings.

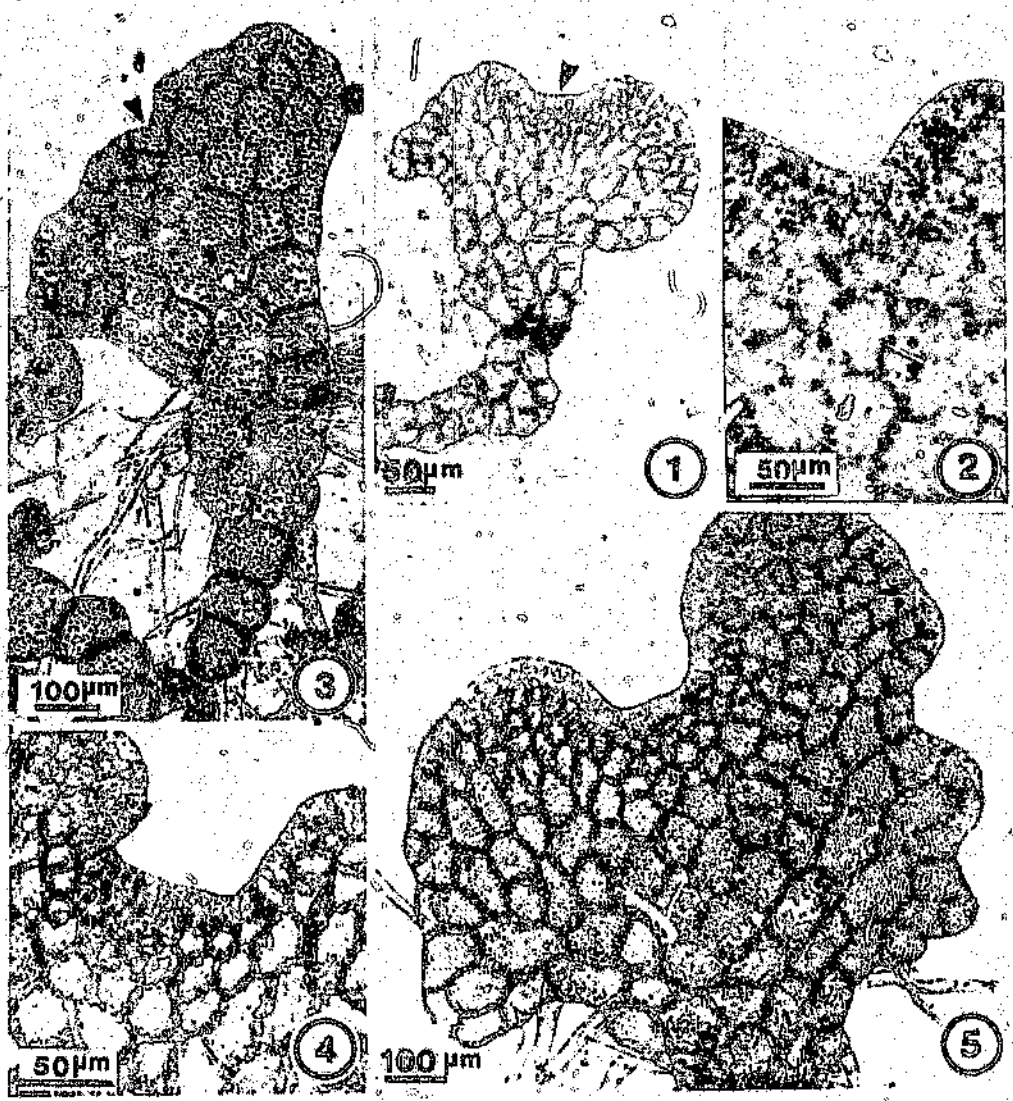

50µm
1
50µm
2
100µm
3
50µm
4
100 µm
5

PLATE 9

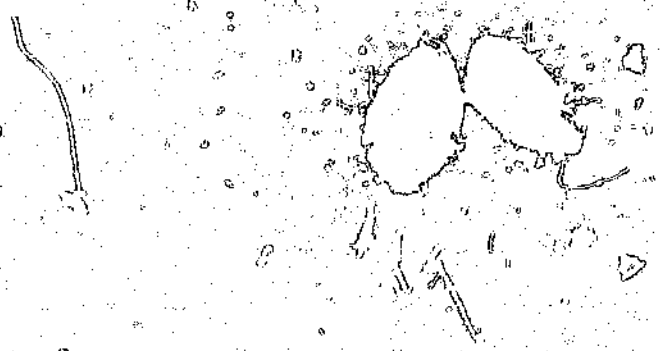
The use of indirect immunofluorescence microscopy to view the microtubular arrangement in the filamentous protballi.

Figure 1:- Autofluorescence of the chloroplasts masks the fluorescence of the microtubule arrays.

Figure 2:- The control slides show a vast amount of autofluorescence caused by the chloroplasts.

Figure 3:- The more basal cells (*) of the long protballi cultured under red light showed little autofluorescence, but were collapsed, therefore, although general microtubular fluorescence could be discerned, no individual microtubular arrays were visualised.

Figure 4:- The apical cell of a long filament showing only autofluorescence of the chloroplasts.



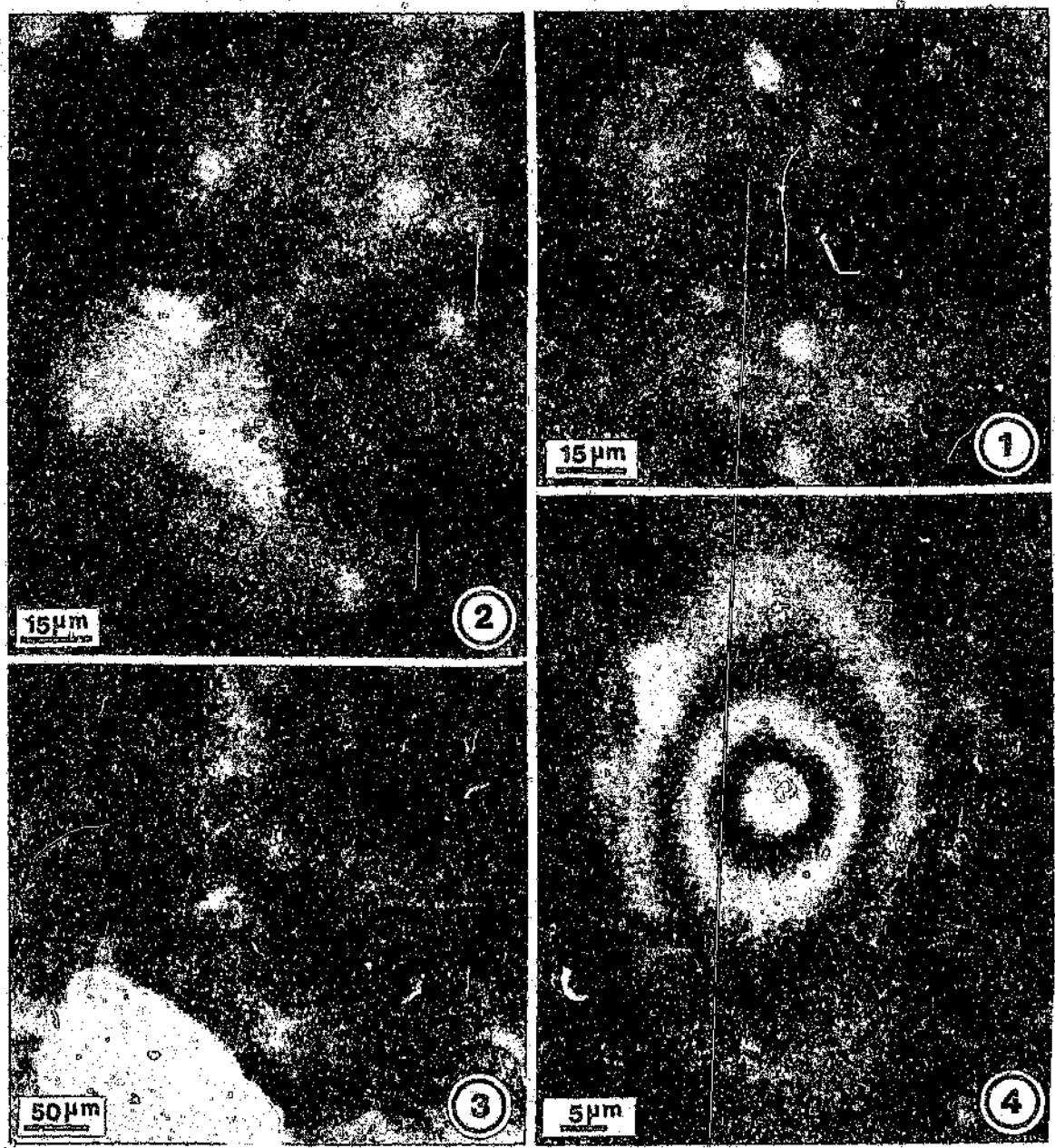

15µm
1
15µm
2
50µm
3
5µm
4

PLATE 10

Ultrastructural details of the vacuole and nucleus of the gametophyte cells of *C. viridis* var. *glauca*.

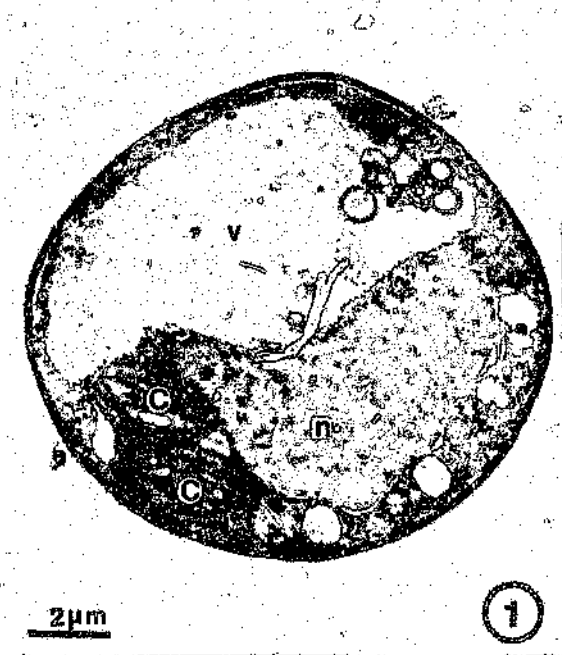
Figure 1:- A cross section through a filamentous prothallus showing the position of the large vacuole (v), the nucleus (n) and numerous chloroplasts (C).

Figure 2:- Gametophyte cells showing the vacuole (v) which is an obvious feature.

Figure 3:- The nucleus (n) showing a granular nucleoplasm with clumped heterochromatin.

Figure 4:- The dark regions within the nucleus are heterochromatin. A glancing section of the nucleolus (arrowhead) can be seen.

Figure 5:- The nucleus (n), surrounded by a double membrane (arrowhead).


V
C
C
n
2µm
1


V
2µm
2


n
1µm
3


n
1µm
4

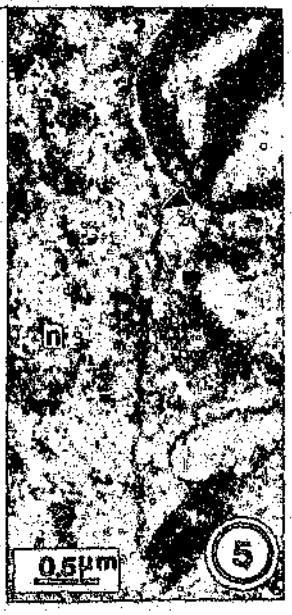

n
0,5µm
5

PLATE 11

The cytoplasmic contents of the gametophyte cells.

Figure 1:- Typical mitochondrial profiles (m) which are commonly seen throughout the cytoplasm.

Figure 2:-Elongated mitochondrion (m), showing the surrounding double membrane and cristae which are somewhat expanded (arrowheads).

Figure 3:- The matrix surrounding the uniformly distributed dictyosomes (d) and mitochondria (m) consists primarily of tubules of endoplasmic reticulum (arrowhead) and numerous small vesicles.

Figure 4:- Layers of endoplasmic reticulum (arrowheads), commonly seen in the cytoplasm of the gametophyte cells show distended cisternae.

Figure 5:- Myelin-like structure (M) found close to the cell wall (cw) in a mature gametophyte cell.

Figure 6:- A myelin-like structure (M) in the cytoplasm of a gametophyte cell.

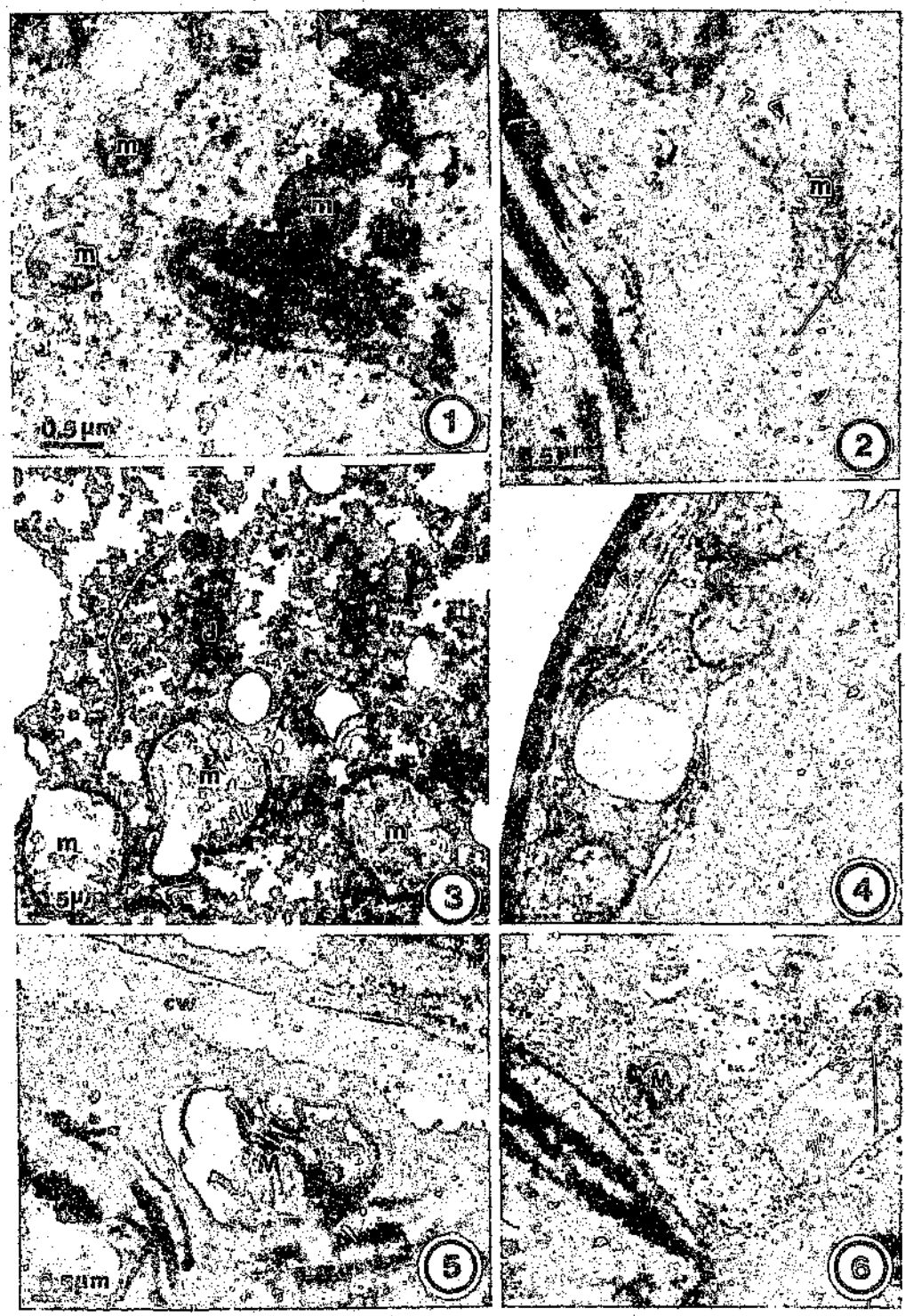

m
m
m
0,5 µm
1
m
2
m
m
m
0,5µm
3
4
cw
M
0,5µm
5
M
6

PLATE 12

The chloroplasts of the gametophyte cells.

Figure 1:- A surface view of gametophyte cells (using Nomarski interference optics) showing the chloroplasts crowded around the cell boundary (arrowhead).

Figure 2:- A section through a filamentous prothallus confirms the chloroplasts' (arrowhead) peripheral arrangement.

Figure 3:- Chloroplasts (C) containing large starch grains (S) and osmiophilic droplets (arrowheads).

Figure 4:- In cross - section the chloroplast profiles (C) are round while in longitudinal section they are elongated.

Figure 5:- A double unit membrane (arrowhead) surrounds the chloroplast - the chloroplast envelope. The double unit membrane lying adjacent to the chloroplast envelope (arrowed) is probably chloroplastic endoplasmic reticulum.

Figure 6:- The thylakoids which occur singly (arrowhead) or aggregated into grana (G).

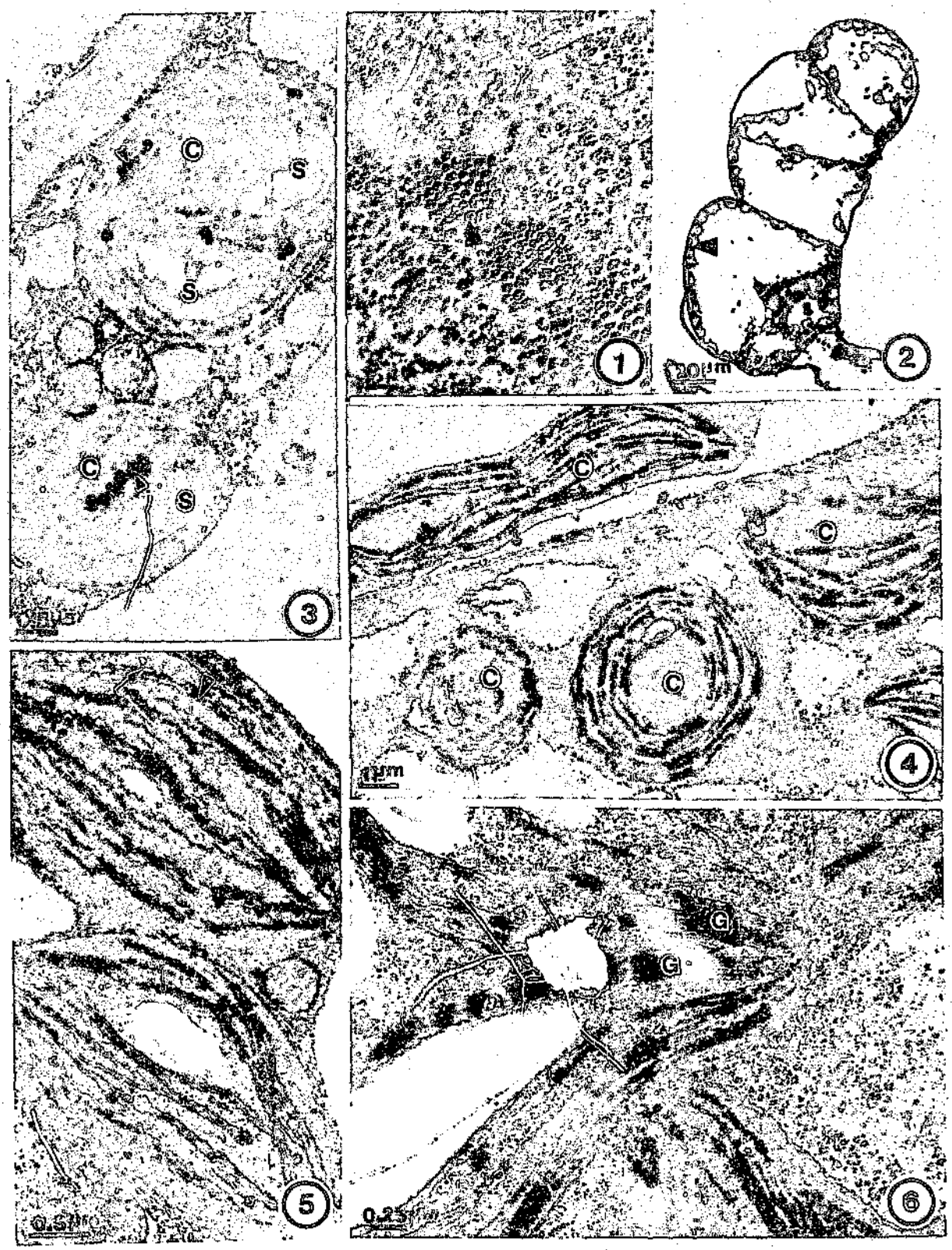

C
S
S
C
S
1
20µm
2
C
C
C
C
1µm
4
0.5µm
5
G
G
0.25µm
6
3

PLATE 13

Ultrastructural details of gametophyte chloroplasts.

Figure 1:- The thylakoids lie in the direction of the longitudinal axis of the chloroplast. Osmiophilic droplets (arrowhead) commonly occur in the stroma of the chloroplast.

Figure 2:- Large starch grains (S) which may occupy a large volume of the mature chloroplasts.

Figure 3:- Triplets of microtubules (arrowed) are seen lying between the chloroplasts (C) and the cell wall (cw) of the gametophyte cells.

Figure 4:- Starch grains (S) and lipid droplets (arrowhead) are obvious features in mature chloroplasts.

Figure 5:- Mitochondria (m) are often seen closely associated with the chloroplasts (C).

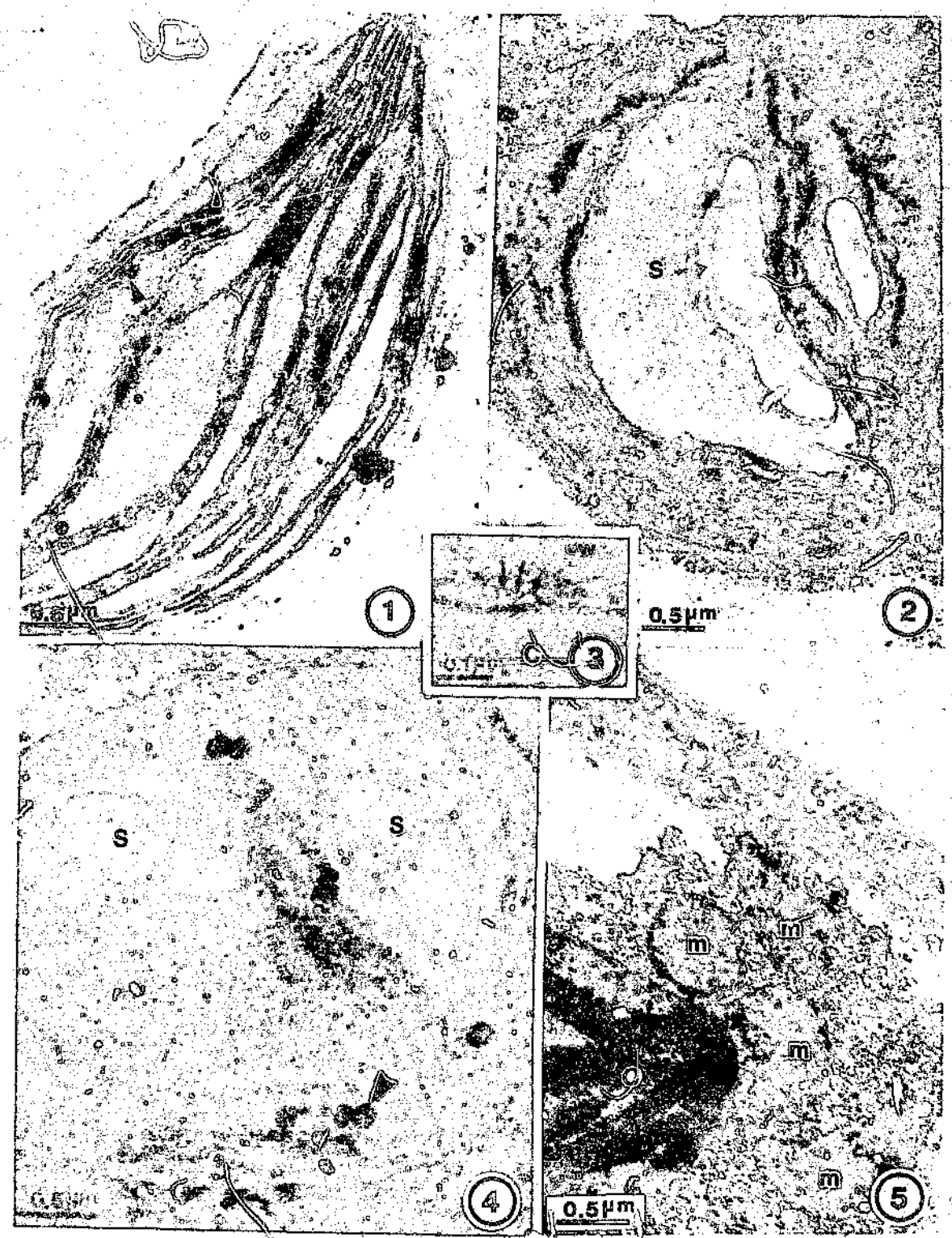

S
0.5µm
1
0.5µm
2
3
S
S
4
m
m
m
m
m
0.5µm
5

PLATE 14

Early stages in the development of the apogamous bud in *C. viridis* var. *glauca*.

Figure 1:- The apical notch of a mature gametophyte.

Figure 2:- The meristematic region at the base of the apical notch (arrowhead) after about forty five days in culture.

Figure 3:- The cells of the meristematic region (arrowhead) which are smaller than those of the surrounding gametophyte tissue.

Figure 4:- A detailed view of the meristem showing the small size of the cells (arrowhead) relative to those of the gametophyte tissue.

Figure 5:- Divisions in the meristem cause a cell plate to be laid down at the base of the apical notch.

Figure 6:- Higher magnification of cell plate.

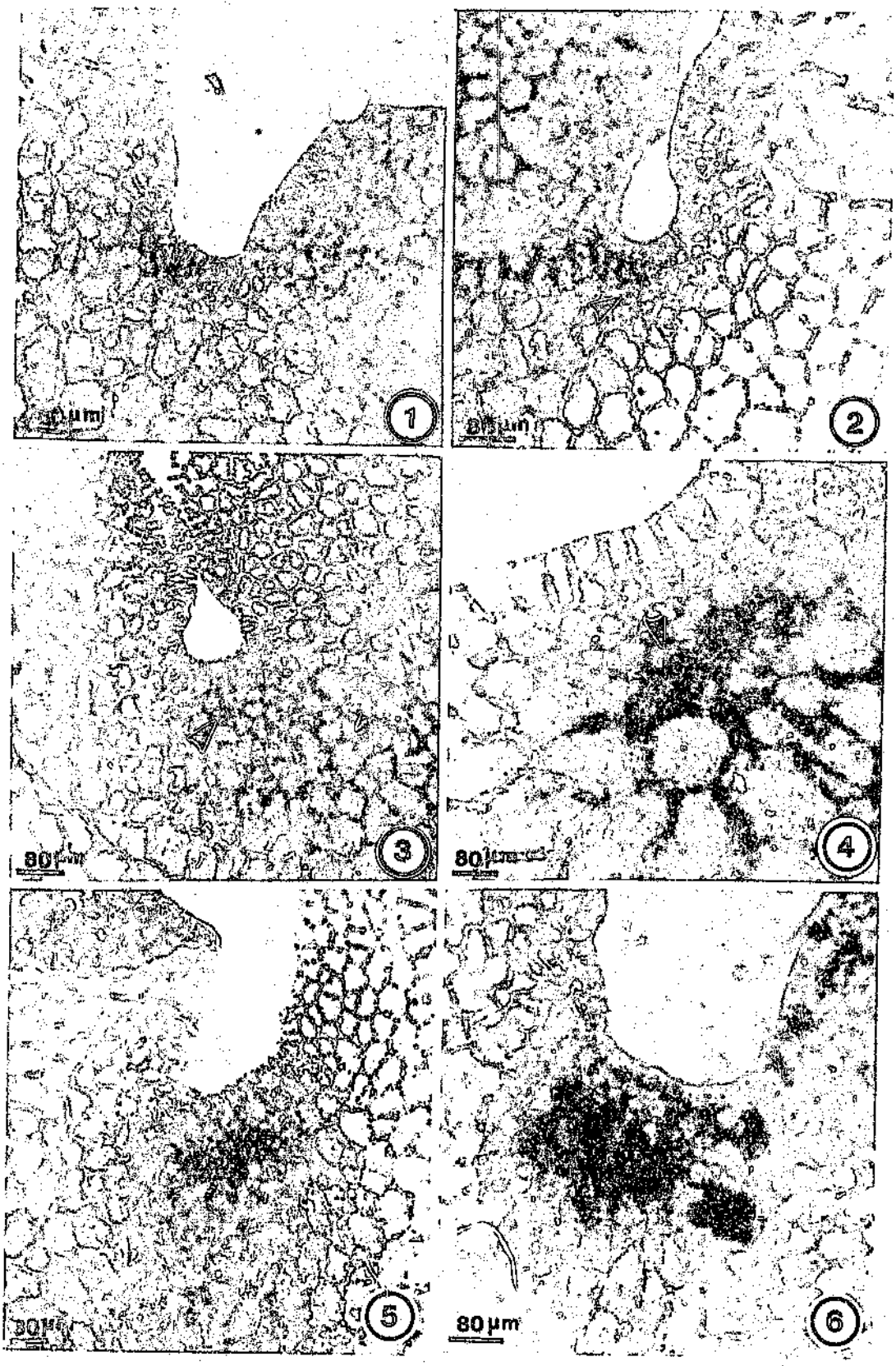

1
2
3
80 µm
4
80 µm
5
80 µm
6

PLATE 15

Development of a mature apogamous bud.

Figure 1:- A dark-green region, the first indication of bud formation, develops at the base of the apical notch.

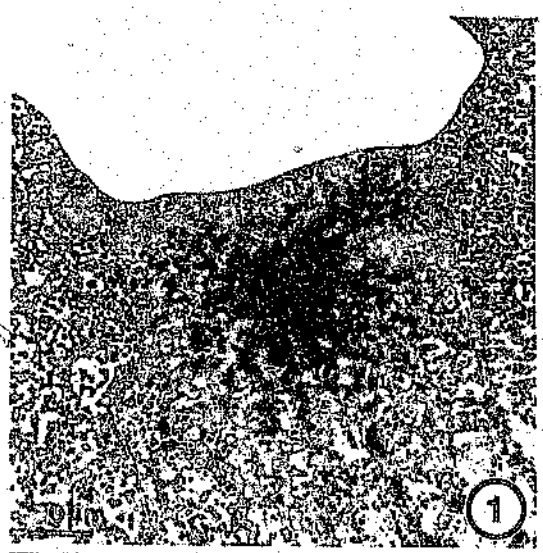
Figure 2:- The thick cushion-like region or apogamous bud which forms as a result of cell divisions at the base of the apical notch.

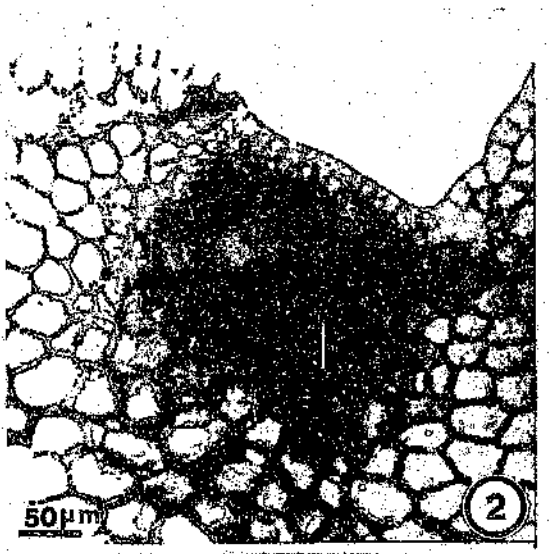
Figure 3:- Multicellular hairs (arrowhead) which develop around the apogamous bud.

Figure 4:- Details of the multicellular hairs (arrowhead) surrounding the developing apogamous bud.

Figure 5:- Increase in size of the apogamous bud is seen with the concomitant increase in number and size of the multicellular hairs.

Figure 6:- A mature apogamous bud (a) with its surface raised above the gametophyte and surrounded by numerous multicellular hairs (arrowhead).


50µm
1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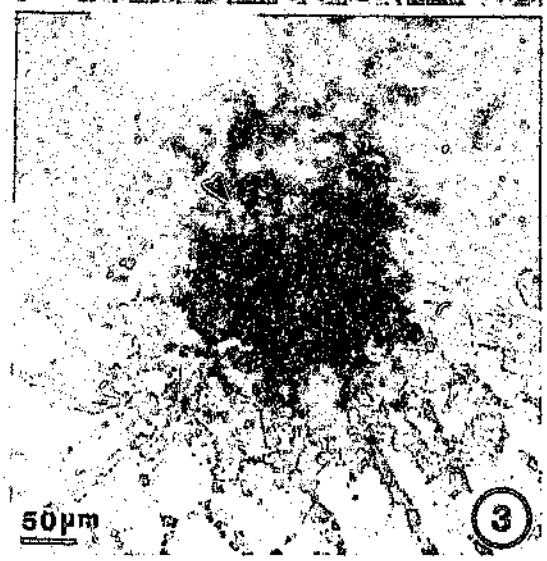
2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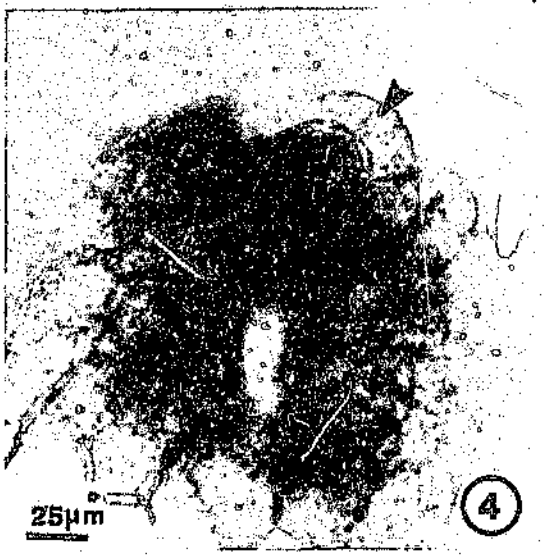
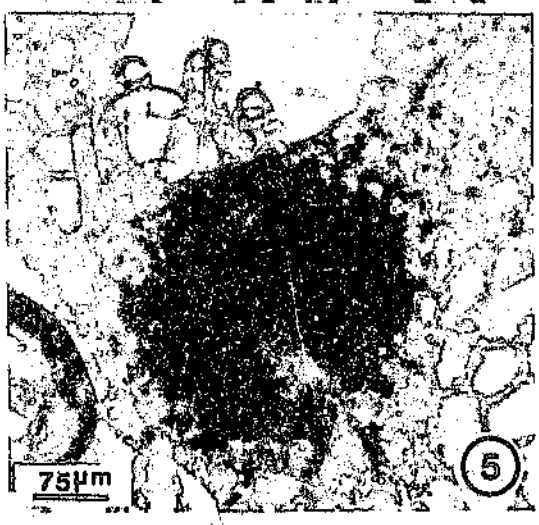
3


25µm
4


75µm
5

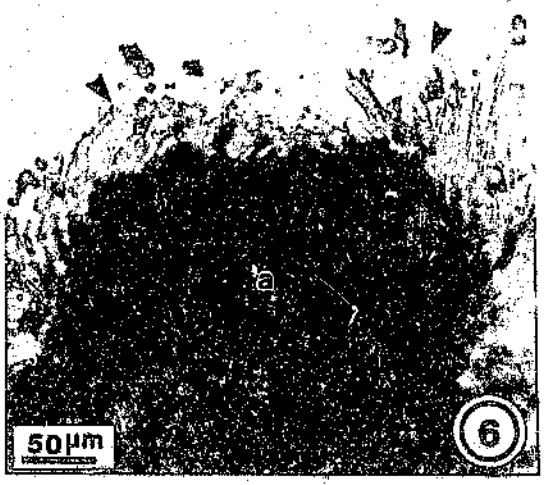

50µm
6

PLATE 16

The appearance of the first leaf from the apogamous bud.

Figure 1:- A mature apogamous bud (a) at the base of the apical notch.

Figure 2:- Root (R) and shoot (s) apices. These differentiate almost simultaneously from the apogamous bud (a).

Figure 3:- The first leaf of the apogamous sporophyte which arises from the apogamous bud after about two months in culture.

Figure 4:- The first leaf which emerges directly from the apogamous bud.

Figure 5:- Elongated structures containing vascular tissue often develop from the apogamous bud.

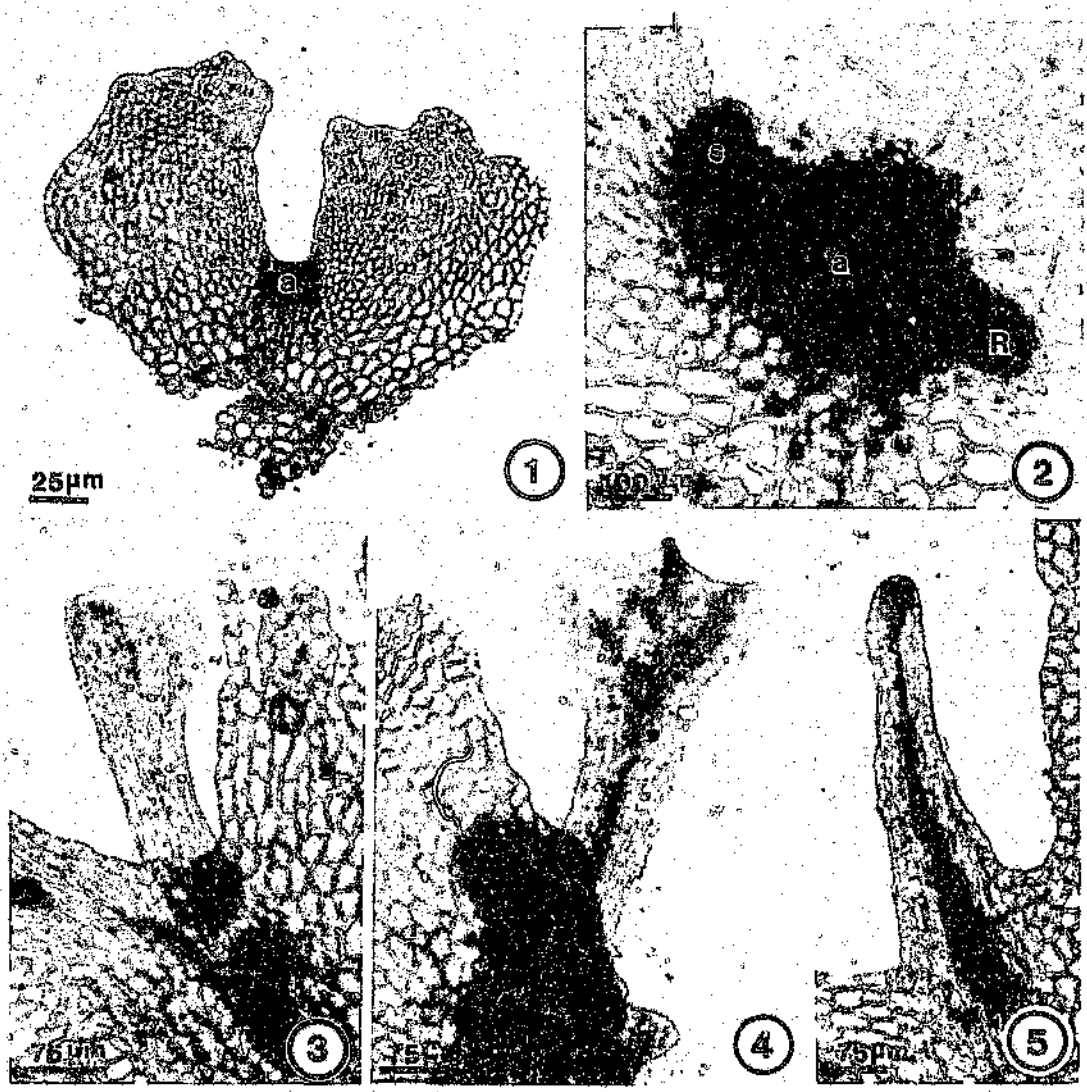

a
25µm
1
a
R
2
75µm
3
75µm
4
75µm
5

PLATE 17

The first leaf and the vasculature of the sporophyte.

Figure 1:- Multicellular hairs (arrowhead) present at the base of the first leaf.

Figure 2:- Multicellular hairs (arrowhead) present along the petiole of the leaf.

Figure 3:- The first leaf which is bilobed. The multicellular hairs (arrowhead) are evident along the petiole of the leaf.

Figure 4:- Multicellular hairs which appear to have glandular tips (arrowhead) are seen on the leaf surface.

Figure 5:- Vascular tissue of the apogamous sporophyte.

Figure 6:- Sectioned annular tracheids (arrowhead), commonly seen in the centre of the leaf petiole.

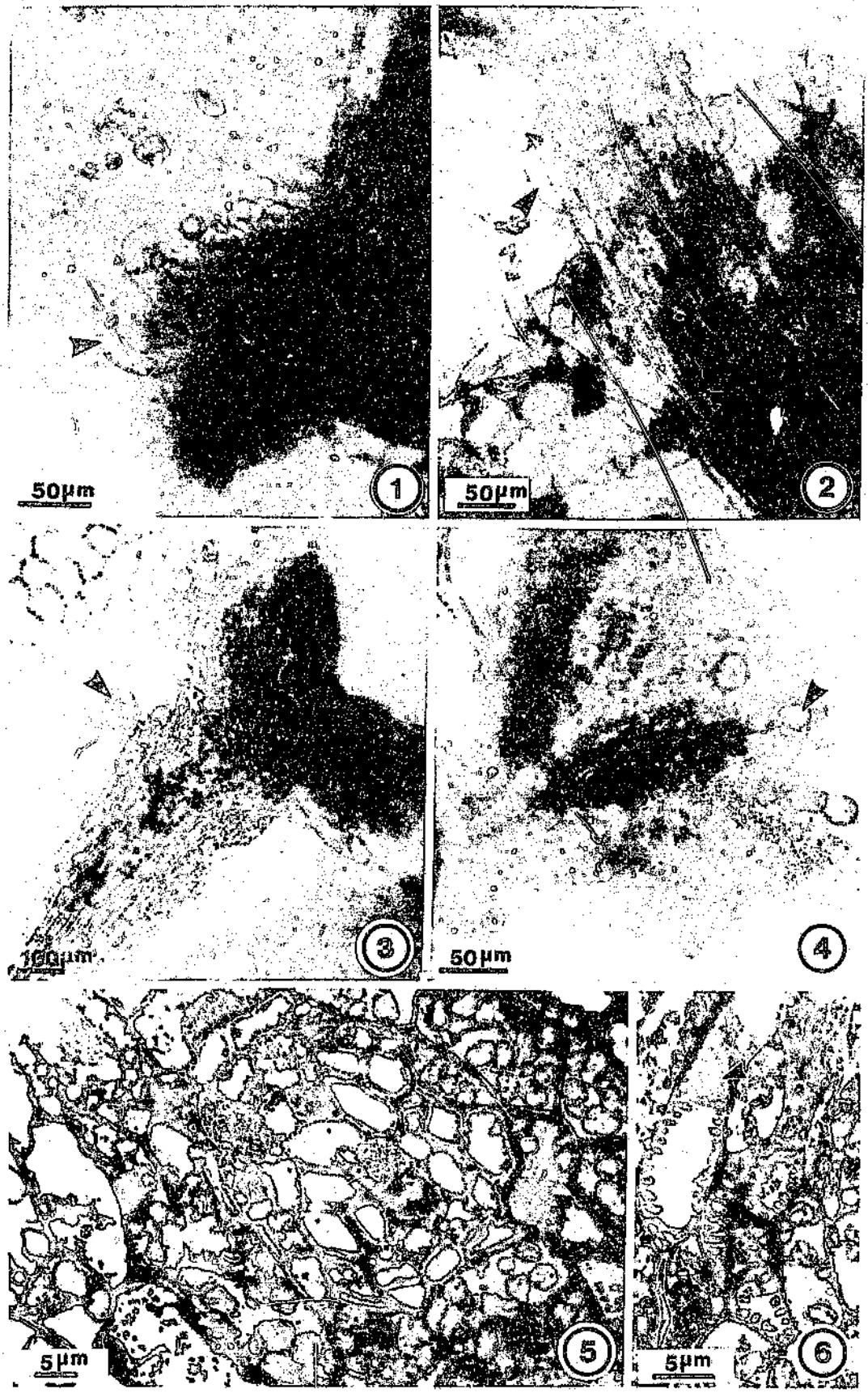

50µm
1
50µm
2
100µm
3
50µm
4
5µm
5
5µm
6

PLATE 18

Development of an independent sporophyte.

Figure 1:- Three well developed leaves held erect over the gametophyte (g).

Figure 2:- The distinct venation of the leaves (arrowhead) indicates the development of a vascular system.

Figure 3:- The root (R) which appears once two to three leaves are well established, with the gametophyte (g) still present but becoming necrotic. Gametophytes at this stage of development are approximately six months old.

Figure 4:- Except for the first leaf, the appearance and development of all subsequent leaves show the characteristic pattern of circination (arrowhead).



0.2mm

1



0.15mm

2



0.2mm

3



0.2mm

4

PLATE 19

Details of the cells comprising the apogamous sporophyte in *C. viridis* var. *glauca*.

Figure 1:- The apogamous bud (a) is embedded in gametophyte tissue (g).

Figure 2:- A longitudinal section through the first leaf of the sporophyte (s). The gametophyte cells (g) are large.

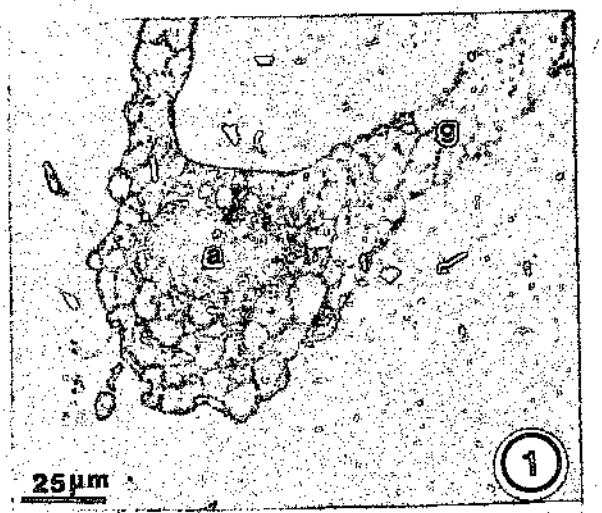
Figure 3:- The cells of the sporophyte have a centrally situated nucleus (n) and a large number of chloroplasts (C).

Figure 4:- The cells of the gametophyte have a large vacuole (v) and peripherally situated chloroplasts and cytoplasm (arrowheads)

Figure 5:- A meristematic region (me) is evident towards the apex of the apogamous sporophytic outgrowth.

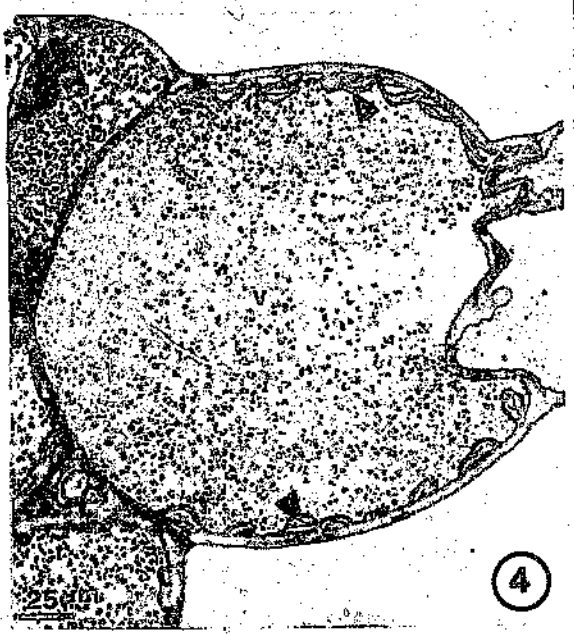
Figure 6:- The nucleus (n) of the meristematic cells (me) occupies the majority of the cell volume.

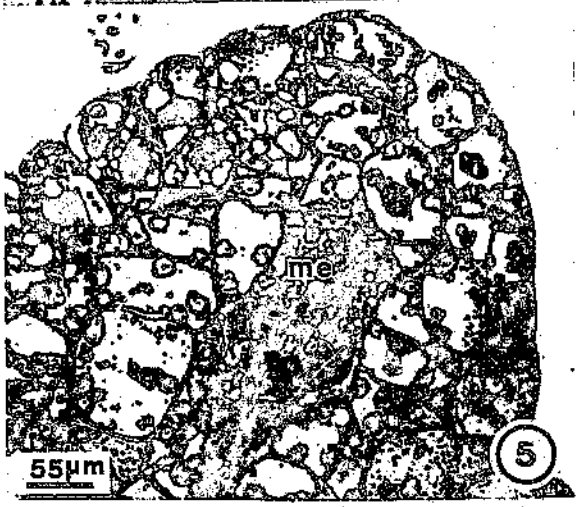
Figure 7:- The cells of the meristematic region (me) of the apogamous region are elongated and smaller than the surrounding sporophytic cells.

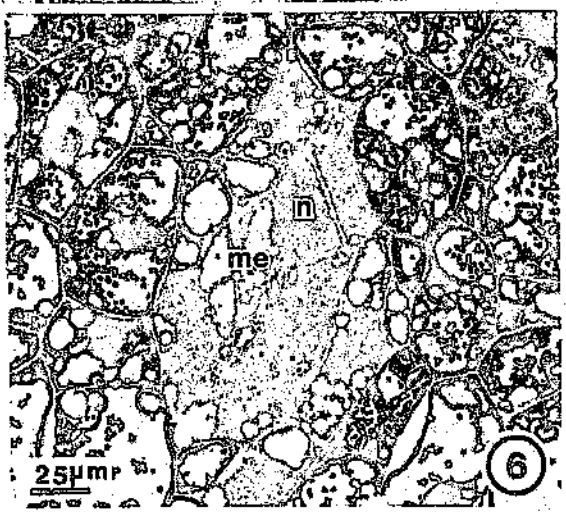

g
a
25µm
1


s
g
55µm
2


n
25µm
3


V
4


me
55µm
5


n
me
25µm
6

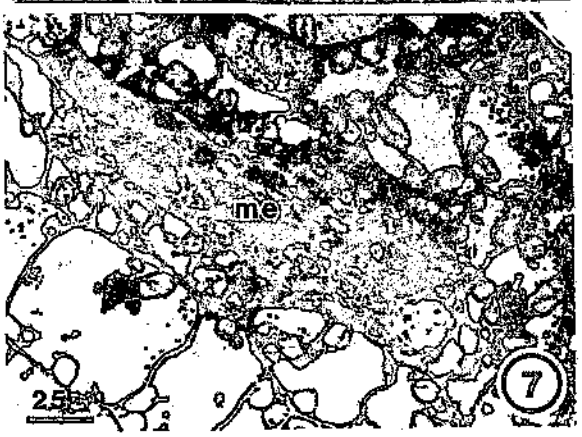

me
7

PLATE 20

Details of the cells at the interface of the sporophytic and gametophytic generations.

Figure 1:- Dark osmiophilic material (arrowheads) present in the vacuoles of the cells forms the boundary between the gametophyte and the apogamous bud.

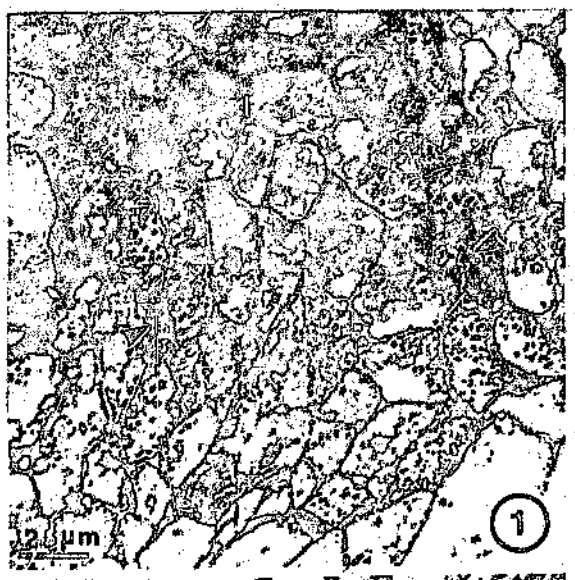
Figure 2:- A distinct boundary between the gametophyte and sporophyte generations which is formed by a darkly staining material (arrowheads) in the vacuoles of the cells occurs at the interface of the two generations.

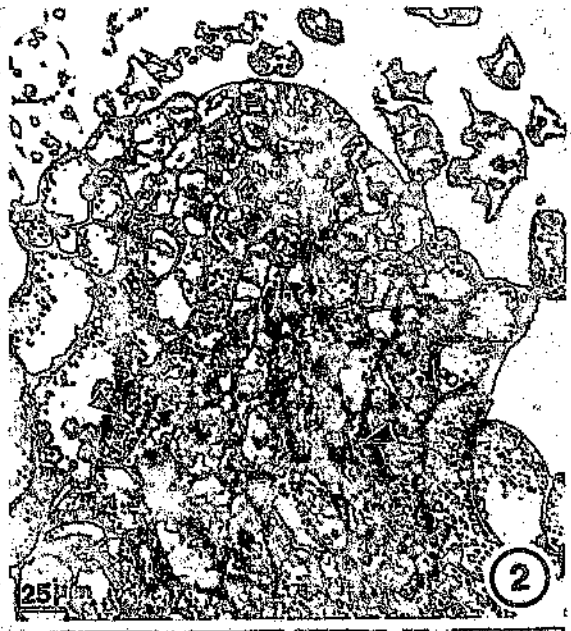
Figure 3:- A meristematic cell (me) of the apogamous sporophyte showing a centrally situated nucleus (n) and vacuoles (v) which are devoid of the osmiophilic layer.

Figure 4:- The arrowhead indicates the presence of darkly staining material in the cells of the apogamous sporophyte.

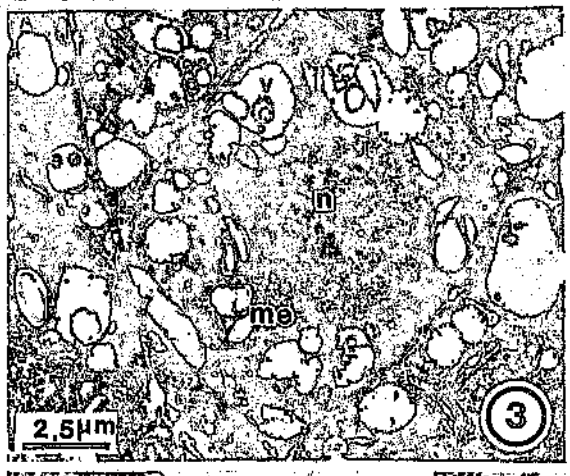
Figure 5:- No darkly staining layer at the level of the tonoplast is noted in the cells of the multicellular hairs (h) which surround the developing sporophyte.

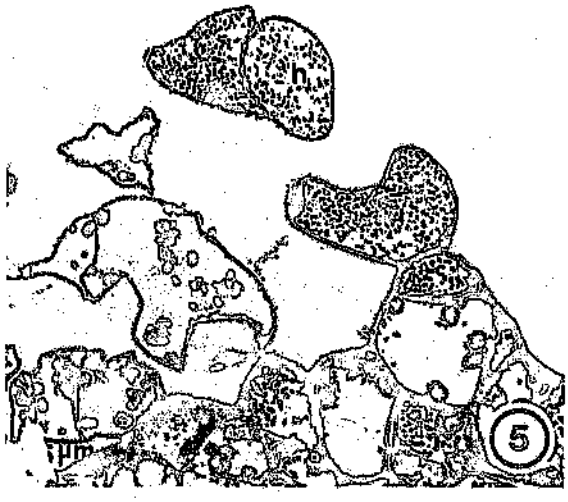
Figure 6:- A thin layer of osmiophilic material (arrowheads) is shown at the level of the tonoplast in the gametophyte cells (g) in the vicinity of the apogamous bud and or sporophyte.


2 µm
1


25µm
2


55µm
4


n
me
2,5µm
3


h
µm
5

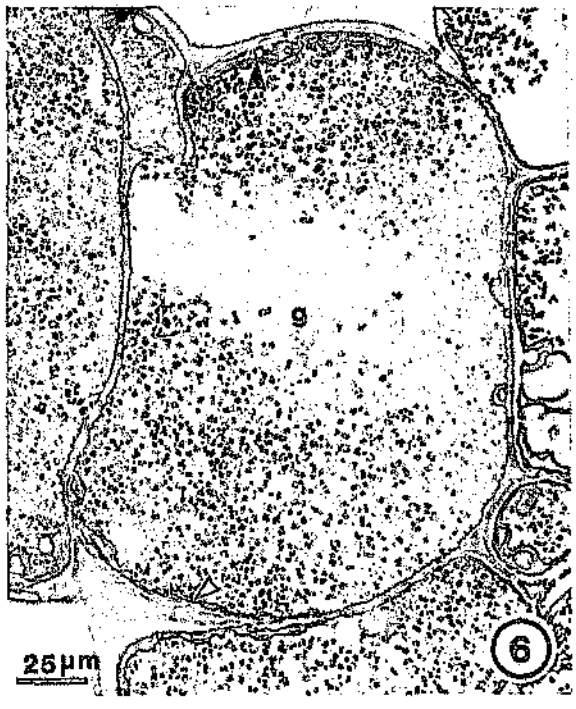

g
25µm
6

PLATE21

The deposition of the osmiophilic layer in the vacuoles of the cells occurring at the interface of the gametophyte and sporophyte generations.

Figure 1:- Small, darkly staining droplets are seen on the tonoplast (arrowheads) in the gametophyte cells close to the apogamous bud.

Figure 2:- A sporophyte cell showing the deposition of osmiophilic material (arrowhead) on the tonoplast.

Figure 3:- The osmiophilic material is clearly laid down on the tonoplast (arrowhead). Some material can be seen within the vacuole.

Figure 4:- Increased deposition of the material on the tonoplast in older cells of the apogamous bud.

Figure 5:- The "droplets" of osmiophilic material are laid down so close to one another that an almost entire layer is formed on the tonoplast (arrowhead). The vacuole (v) is packed with granular material which may condense on the tonoplast to form the layer.

Figure 5:- A detailed view of the layer formed at the tonoplast of a mature cell in the apogamous bud.

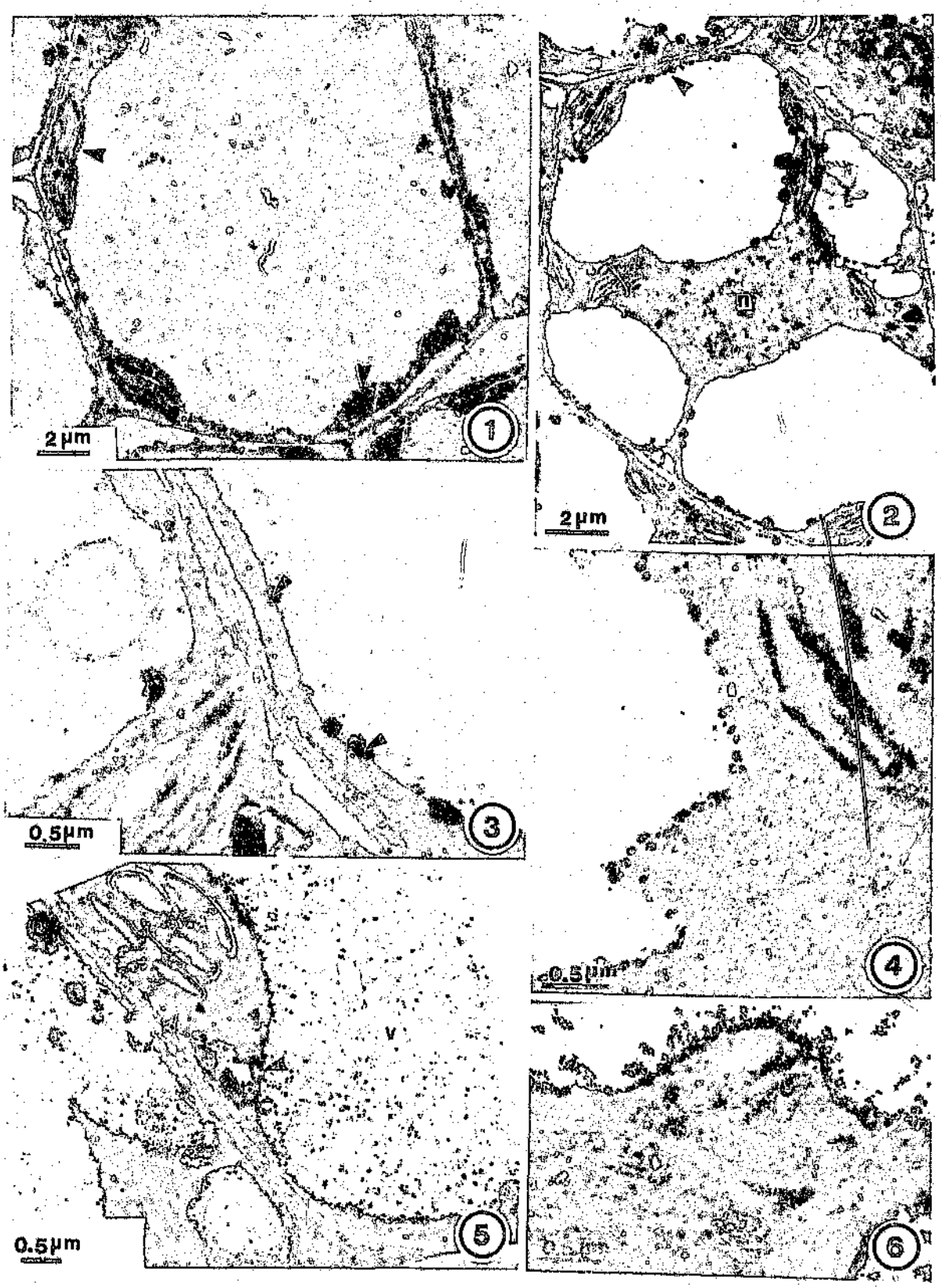

2 µm
1
n
2 µm
2
0,5µm
3
0.5µm
4
V
5
6

PLATE 22

The presence of the osmiophilic layer separates the gametophyte and sporophyte generations in *C. viridis* var. *glauca*.

Figure 1:- The arrowheads indicate the presence of a thick osmiophilic layer surrounding the vacuole of mature cells of the apogamous bud.

Figure 2:- A boundary which forms between the sporophyte (s) and gametophyte (g) generations is seen due to the presence of peripheral osmiophilic bodies in the vacuole of these cells.

Figure 3:- Mature apogamous sporophyte cells with their vacuoles (v) filled with granular material and a distinct layer present at the level of the tonoplast (arrowheads). The plastids (*) in these cells filled with compound starch grains.

Figure 4:- The gametophyte tissue (g) and sporophyte tissue (s) are easily discernible due to the presence of cells with a thick tonoplastic layer which form a boundary between the two generations.

Figure 5:- The osmiophilic layer (arrowheads) is prominent in two - three layers of cells lying between the typical sporophyte (s) and gametophyte (g) cells.

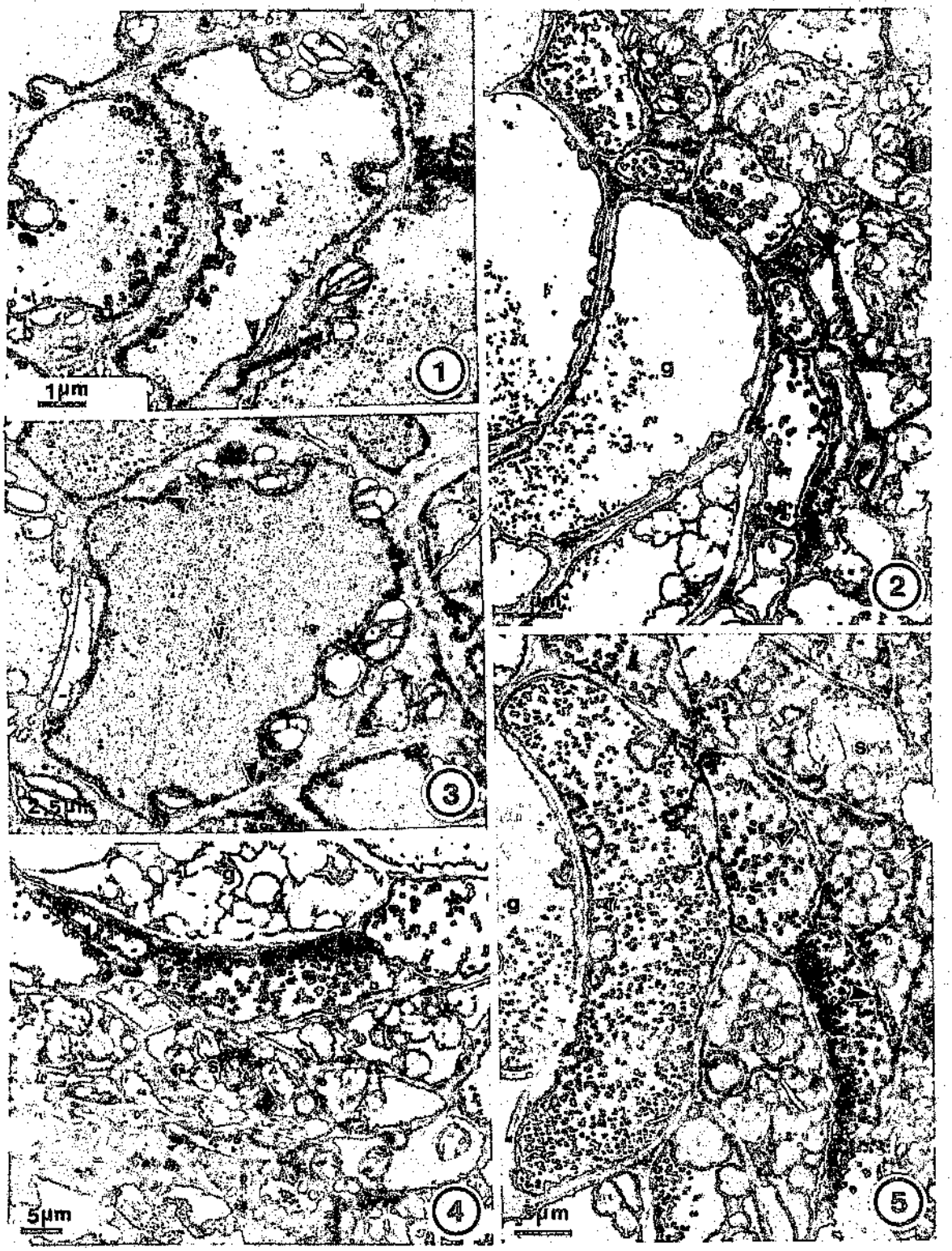

1µm
1
g
2
2,5µm
3
g
s
5µm
4
s
g
µm
5

PLATE 23

Histochemical identification of the osmiophilic particles which forms the layer on the tonoplast of cells separating the gametophyte and sporophyte generations.

Figure 1:- The particles within the vacuole (arrowhead) stain deep blue with toluidine blue.

Figure 2:- The starch grains (arrowhead) within the chloroplasts stained pink with the periodic acid-Schiff's (PAS) staining reaction (They appear red in this photograph due to the use of Nomarski interference optics). The nuclei and particles within the vacuole did not stain at all.

Figure 3:- The proteins in the nuclei stained blue (arrowhead) with the aniline blue black while the starch in the chloroplasts took up the PAS stain and appeared pink/red (arrowhead) when viewed under bright field. The particles in the vacuole were unstained.

Figure 4:- The combination of the PAS and aniline blue black staining techniques stained the starch in the chloroplasts (*) deep purple and the nuclei blue (arrowed), but the particles within the vacuoles remained unstained (arrowhead). These colour reactions were observed using Nomarski interference optics.

Figure 5:- Sections stained with PAS and aniline blue black showed the starch in the chloroplasts to stain a maroon to deep red (large arrowhead) and the nuclei to stain blue (arrowed), while the particles within the vacuole (arrowhead) did not take up either of the stains.

Figure 6:- Periodic acid Schiff (PAS) control slide showing no staining of the starch in the chloroplasts. The nuclei stained blue (arrowed) with the aniline blue black.

Figure 7:- A control section for the aniline blue black staining technique showing no staining of any of the organelles when viewed with Namaski interference optics.

Figure 8:- Bright field observation of an aniline blue black control slide showing that no organelles stain and the particles in the vacuole (arrowhead) reflect the light.

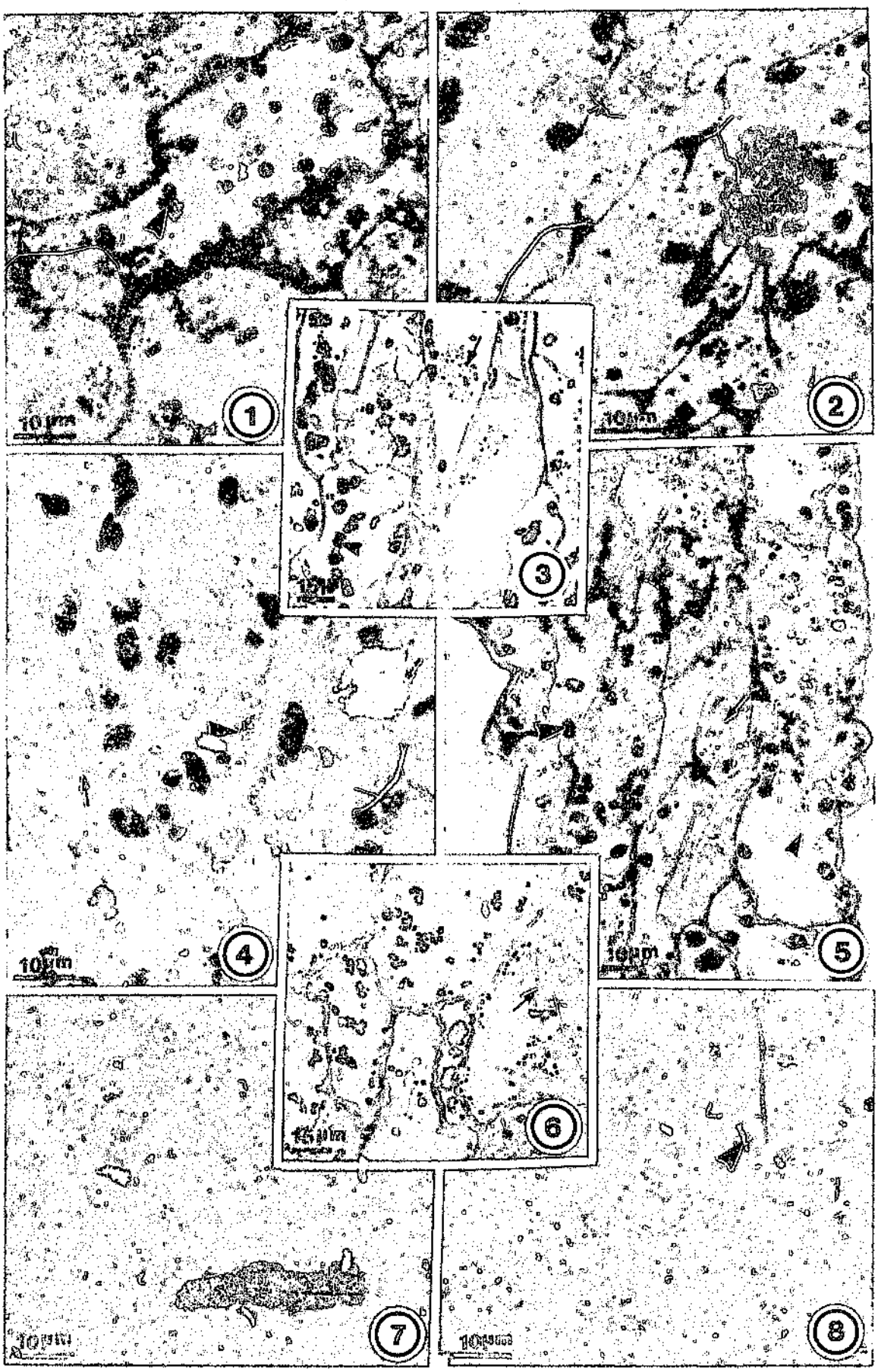

10 μm
1
10 μm
2
3
10 μm
4
10 μm
5
6
10 μm
7
10 μm
8

PLATE 24

Safranin fast green and Sudan Black B staining reaction of the layer in the developing apogamous bud of *C. viridis* var. *glauca*.

Figure 1:- The layer-forming particles (arrowhead) stain a brownish - red when treated with the safranin and fast green.

Figure 2:- With Nomarski interference optics the particles within the vacuoles appear purple-blue (arrowhead). The starch within the chloroplasts stains blue and are highlighted by this staining technique.

Figure 3:- The particles within the vacuole (arrowhead) of the cells surrounding the developing apogamous bud stain pink/red with the Safranin fast green staining technique.

Figure 4:- Using Normarski interference optics, the particles within the vacuole stain pale pink (arrowhead) when treated with Sudan Black B.

Figure 5:- At higher magnification it can be seen that the particles within the vacuole (arrowhead) stain pale pink with Sudan Black B. The chloroplasts (*) appear cerise/purple due to prior staining with PAS.

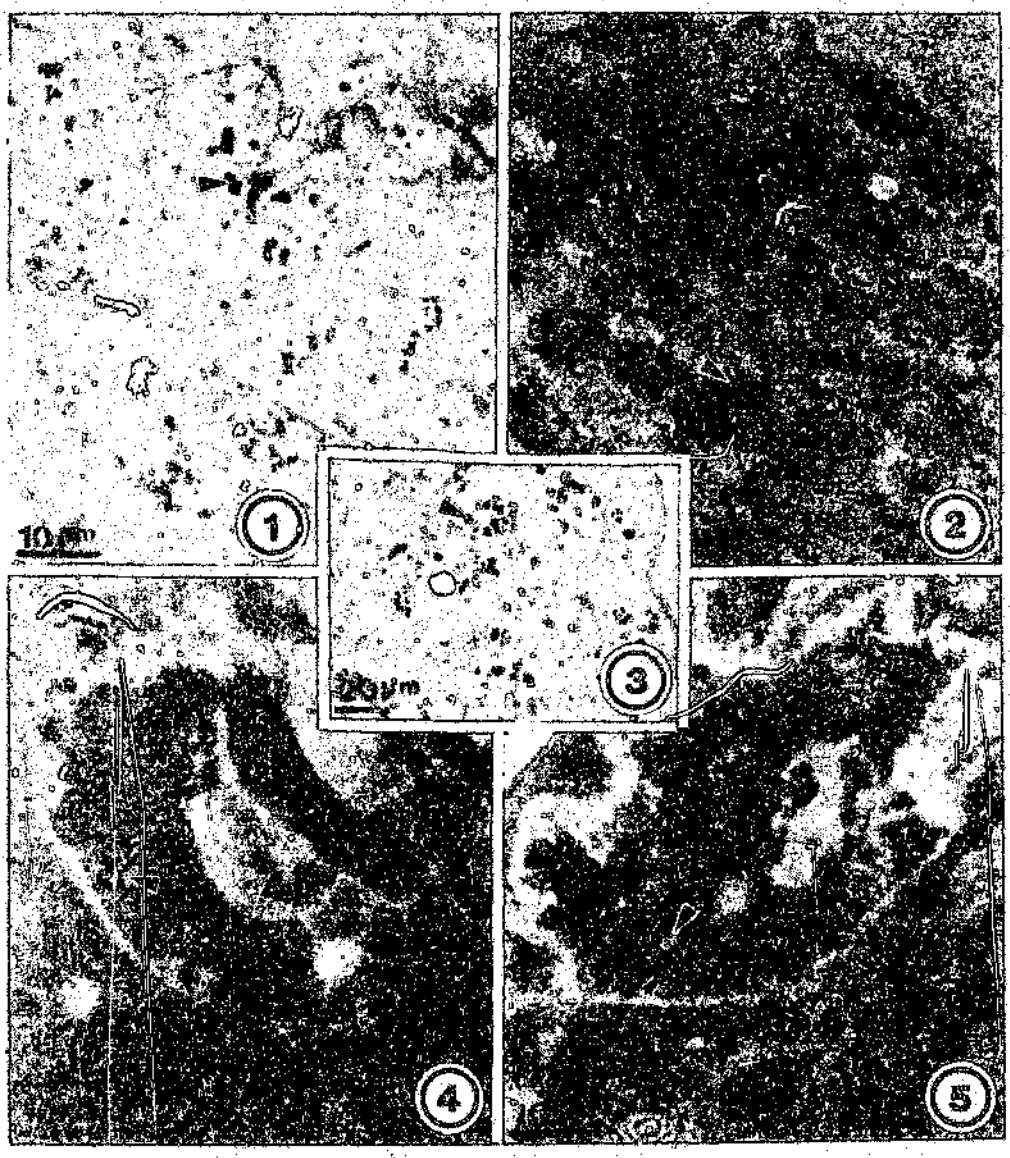

10 µm
1
2
3
4
5

PLATE 25

Chromosome spreads and nuclei of gametophyte and sporophyte tissues.

Figure 1:- Chromosome spread in a prothallial cell.

Figures 2-4:- A through f[illegible]s of a gametophyte cell at high power showing a chromosome spread. The chromosomes were counted from tracings from these photographs.

Figure 5:- Nuclei of gametophyte cells. The nucleus on the left (arrowhead) is undergoing division.

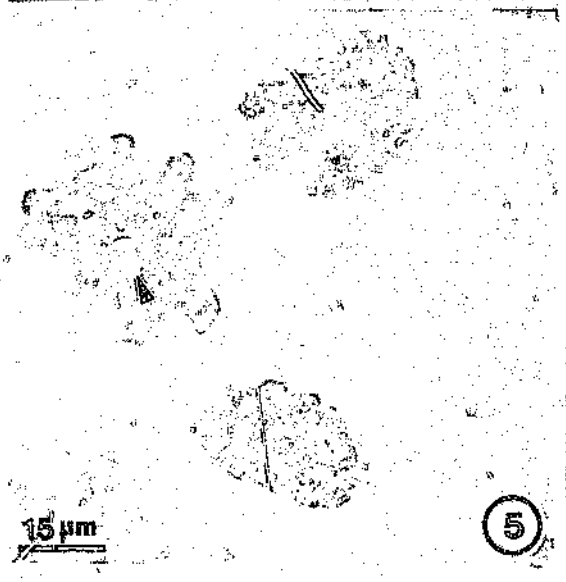
Figure 6:- Nucleus of a cell from the first leaf of the sporophyte.

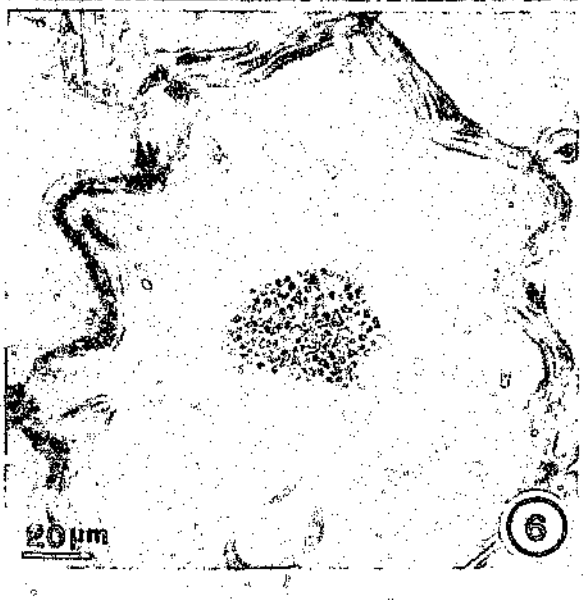

10 µm
1


10 µm
2


10 µm
3


10 µm
4


15 µm
5


20 µm
6

APPENDIX II

BUFFERS

Blocking Solution (pH 7.4)

20mM Tris - HCl	2,42g
200mM NaCl	11,69g
0.5mg/ml $NaBH_4$	0,5g
0.75% (w/v) BSA	7,5g
0.1% (w/v) Gelatin	1,0g
Distilled Water	1000ml

EGTA Buffer

5mM EGTA	0,07g
2% (w/v) Driselase	1,0g
50μg/ml Leupeptin	2,5mg
5% (w/v) Mannitol	2,5g
Distilled Water	50ml

Microtubule Stabilising Buffer (MTSB) (pH 6.8)

0.1M PIPES - KOH	30,24g
1mM $MgCl_2$	0,21g
5mM EGTA	1,46g
0.01% (v/v) Triton X 100	0,1ml
Distilled Water	1000ml

Stock solutions:-

A) Monobasic sodium phosphate 27,8g

Distilled Water 1000ml

B) Dibasic sodium phosphate

($NA_2HPO_4.7H_2O$ 53,65g

or $NA_2HPO_4.12\ H_2O$ ..... 71,7g)

Distilled Water 1000ml

For a buffer solution with a pH of 6.9

Stock solution A 45ml

Stock solution B 55ml

EMBEDDING PROTOCOL FOR ELECTRON MICROSCOPY

Table 2: The Infiltration of Fern Gametophytes with Spurr's Resin.

Alcohol (%)	Spurr's (%)	Time (hr)
75	25	2
50	50	16
25	75	6
	100	16
	100	6

Spurr's Resin (Spurr, 1961)

Add in order:-

ERL 4206 ..... 10,0g

DER 736 ..... 6,0g

NSA 26,0g

NSA 26,0g

S-1 ..... 0,4g

Leave on a magnetic stirrer for half an hour before use. If refrigerated it has a shelf life of three to four days.

FIXATIVES

Farmer's Fixative

100% alcohol 300ml

Glacial acetic acid 100ml

NUTRIENT MEDIA

Bolds Basal Medium (pH 6.6)

Stock solutions:-

A) Macronutrients

$NaNO_3$ 10g

$CaCl_2$ 1,0g

$MgSO_4.7H_2O$ 3,0g

K_2HPO_4 ..... 3,0g

KH_2PO_4 ..... 7,0g

NaCl 1,0g

Distilled water 400ml

B) EDTA

EDTA 50g

KOH 31g

Distilled water 1000ml

C) IRON

$FeSO_4.7H_2O$ 4,98g

H_2SO_4 ..... 1,0ml

Distilled water 1000ml

D) BORON

H_3B_{O3} 11,42g

Distilled water 1000ml

E) MICRONUTRIENTS

$ZnSO_4.7H_2O$ 8,82g

$MnCl_2.4H_2O$ 1,44g

MoO_3 ..... 0,71g

$CuSO_4.5H_2O$ 1,57g

$Co(NO_3)_2.6H_2O$ 0,49g

Distilled water 1000ml

Culture solution:-

Stock solution A 10ml

Stock solution B 1ml

Stock solution C 1ml

Stock solution D 1ml

Stock solution E 1ml

Distilled water 986ml

Knops Solution (pH 5.2 - 6.3)

$Ca(NO_3)_2.4H_2O$ 0,8g

KNO_3 ..... 0,2g

KH_2PO_4 ..... 0,2g

$MgSO_4.7H_2O$ 0,2g

Nitsch Tace Elements 1ml

Ferric Citrate 1ml

Distilled water 1000ml

Knudsons Mineral Salt Solution (pH 5.5 - 6.3)

$Ca(NO_3)_2.4H_2O$ 0,5g

$(NH_4)_2SO_4$ 0,25g

$MgSO_4.7H_2O$ 0,125g

K_2HPO_4 ..... 0,125g

Nitsch Trace Elements 1ml

Ferric citrate 1ml

Distilled Water 1000ml

Modified Mehr's Solution (pH 5.8)

Stock Solutions:-

A) $MgSO_4$ 51,0g

KNO_3 ..... 12,0g

Distilled water 1000ml

B) $FeCl_3.6H_2O$ 1,7g

Distilled water 1000ml

C) $Ca(NO_3)_2.H_2O$ 144,0g

Distilled water 1000ml

D) KH_2PO_4 ..... 12,5g

K_2HPO_4 ..... 12,5g

Distilled water 1000ml

E) Mycostatin ..... 1000 units

Distilled water 1000ml

Culture solution:-

Stock solution A	10ml
Stock solution B	10ml
Stock solution C	10ml
Stock solution D	10ml
Stock solution E	10ml
Distilled water	950ml

Moores Medium (pH 5.5 - 6.5)

NH_4NO_3	0,5g
KH_2PO_4	0,2g
$MgSO_4.7H_2O$	0,2g
$CaCl_2.2H_2O$	0,1g
Nitsch Trace Elements	1ml
Ferric Citrate	0,05g
Distilled water	1000ml

Nitsch Trace Elements

A) Ferric citrate	10mg
Distilled water	1000ml
B) H_2SO_4 sp. grav 1.83	0,5ml
$MnSO_4.4H_2O$	3,0g
$ZnSO_4.7H_2O$	0,5g
H_3BO_3	0,5g
$CuSO_4.5H_2O$	0,025g
$Na_2MoO_4.2H_2O$	0,025g
Distilled water	1000ml

Provosoli Enrichment Medium (pH 7.5 - 8.0)

Stock solutions:-

A) $NaNO_3$ 35g

Distilled water 100ml

B) Na_2glycerophosphate 5g

Distilled water 100ml

C) Vitamin B_{12} 1,0mg

Distilled water 100ml

D) Thiamine 50mg

Distilled water 100ml

E) Biotin 0,5mg

Distilled water 100ml

F) Tris Buffer

Fe (as EDTA 1:1 molar)

$Fe(NH_4)_2(SO_4).6H_2O$ 351mg

Na_2EDTA 300mg

Distilled water 500ml

G) PII Trace Metals

H_3BO_3 ..... 1,14g

$FeCl_3.6H_2O$ 49mg

$MnSO_4.4H_2O$ 164mg

$ZnSO_4.7H_2O$ 22mg

$CoSO_4.7H_2O$ 4,8mg

Na_2EDTA 1,0g

Distilled water 1000ml

H) Stock solution A 8ml

Stock solution B 8ml

Stock solution C 8ml

Stock solution D 8ml

Stock solution E 8ml

Distilled water 1000ml

I) Stock solution H 200ml

Stock solution F 200ml

Stock solution G 200ml

Distilled water 650ml

Culture solution:-

Stock solution I 20ml

Distilled water 1000ml

STAINS FOR ELECTRON MICROSCOPY

Lead Citrate

Stock solutions:-

A) Trisodium citrate 37,7g

Distilled water 100ml

B) Lead nitrate 33,1g

Distilled water 100ml

C) 1N NaOH 49g

Distilled water 100ml

Final staining solution:-

Add in order

Distilled water 16ml

Solution A 3ml

Stir well.

Solution B 2ml

Stir to homogenise.

Solution C 4ml

Swirl until precipitate dissolves.

Uranyl Acetate

Uranyl acetate 1g

Double distilled water 50ml

95% ETOH 1ml

Store in a dark bottle in the refrigerator.

STAINS FOR LIGHT MICROSCOPY

Deamination Fluid (Jensen, 1962)

60% $NaNO_3$ 20ml

1% Acetic Acid 60ml

Fast Green

Fast Green FCF 0,1g

Distilled Water 100ml

Feulgen's Reagent Johansen (1940) (pH 2,2)

Basic fuchsin 0,7g

Sodium metabisulphate 3,8g

0,15N HCl 200ml

Place mixture on a magnetic stirrer for two to three hours at room temperature. Decolourise with 1g activated charcoal by shaking vigorously for one to two minutes. Filter and make up to 200ml with distilled water. Store in a tightly stoppered bottle. (Solution must be colourless).

Safranin (Johansen, 1940).

Safranin 4g

Methyl cellusolve 200ml

95% alcohol 100ml

Distilled water 100ml

Sodium acetate 4g

Formalin 8ml

Store in a refrigerator.

Schiffs Reagent

Distilled water 100ml

Basic Fuchsin 0,5g

Boil the water and allow to cool to about 70°C before adding the dye. Dissolve by shaking. Cool the solution to about 25°C and add

Sodium or Potassium metabisulphite 0,5g

1N HCl 10ml

Plug the neck of the flask tightly with cottonwool and allow to stand overnight. Add

Decolourising charcoal 2g

and shake well. Allow the solution to stand for thirty minutes before filtering. The colourless solution should be kept in a tightly stoppered bottle and stored in a refrigerator. The solution may be used over a period of several weeks as long as the solution remains colourless.

Sudan III (Jensen, 1962)

Sudan III is dissolved in excess in 70% alcohol. It is kept for one week at 50°C and then filtered before use. It may be used for several months as long as it is kept in a tightly stoppered bottle to prevent evaporation occurring.

Toluidine Blue

Method used by the Electron Microscope Unit, University of the Witwatersrand.

Stock Solutions:-

A) 1% Sodium Tetraborate 1,0g

Distilled water 99ml

B) 1% Toluidine Blue 1,0g

Stock solution A 99ml

C) 1% Pyronin G 1,0g

Distilled water 99ml

Staining solution:-

To use for general staining of resin sections for light microscope observations take 1 part solution B and add 4 parts solution C.

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Name of thesis: Gametophyte Development In Cheilanthes Viridis Var. Glauca (adiantaceae) With Special Reference ToApogamy.

PUBLISHER:
University of the Witwatersrand, Johannesburg
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